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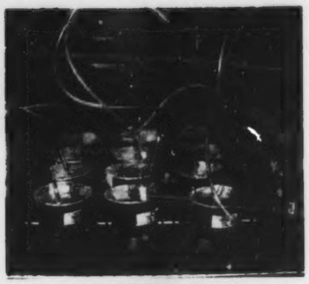

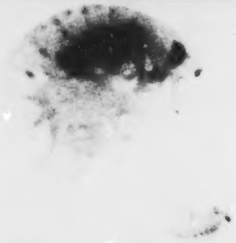

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Biological Test Method: Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*

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Biological Test Method: Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*

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Abstract

Methods recommended by Environment Canada for performing sediment toxicity tests using the freshwater amphipod Hyalella azteca *are described in this report. The endpoints for the test are survival and weight of amphipods at the end of a 14-day assay. The test is intended primarily for measuring the adverse effect(s) of freshwater sediments, although procedures for testing estuarine sediments (≤15 ‰ salinity) are also described.*

This 14-day sediment toxicity test is conducted at 23 ± 1 °C in 300-mL glass beakers or jars containing a 100-mL layer of sediment and 175 mL of overlying water. A minimum of five test chambers, each containing 10 H. azteca, *are normally used to replicate each treatment. The test may be run as a single-concentration assay (e.g., using undiluted samples of field-collected sediment), or as a multi-concentration assay (e.g., a spiked-sediment test with chemical/sediment mixtures) to determine the threshold of effect. Amphipods are 2 to 9 days old at the start of the test.*

The test may be performed either as an intermittent-renewal assay, or as a static (i.e., no renewal) toxicity test. If the renewal option is chosen, twice daily renewal of the overlying water in each test chamber is required, and the overlying water is normally not aerated. If the static option is chosen, the overlying water is aerated. Using either test option, the animals are fed a mixture of yeast, Cerophyll™, and trout chow (YCT). An aqueous suspension of YCT is added to each test chamber, either as a daily inoculum of 1.5 mL (equivalent to ~2.7 mg dry weight), or three times per week on nonconsecutive days using an inoculum of 3.5 mL (~6.3 mg dry weight). Selection of either option depends on the objectives of the study and perhaps also on regulatory guidelines or requirements.

General or universal conditions and procedures are outlined for test preparation and performance. Additional conditions and procedures are stipulated that are specific to the intended use of the test. This test is suitable for measuring and assessing the toxicity of samples of field-collected sediment, sludge, or similar particulate material, or of sediment spiked (mixed) in the laboratory with chemical(s) or chemical substance(s), contaminated sediment, or other particulate material. Instructions and requirements are included on test facilities, sample collection, handling and storing samples, culturing H. azteca, *preparing sediment or spiked-sediment mixtures and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculation, and the use of reference toxicants.*

Résumé

Le présent rapport décrit les méthodes recommandées par Environnement Canada pour exécuter les épreuves de détermination de la toxicité des sédiments à l'aide de l'amphipode dulcicole Hyalella azteca. *Les paramètres de mesure sont la croissance et la survie des amphipodes au cours des 14 jours de durée de l'épreuve. Cette dernière vise principalement à mesurer le ou les effets nocifs des sédiments des milieux d'eau douce. On décrit également un mode opératoire pour les sédiments estuariens (salinité ≤ 15 ‰).*

L'épreuve est réalisée dans des bechers ou des jarres de verre de 300 ml renfermant 100 ml de sédiment recouvert de 175 mL d'eau et maintenus à la température de 23 ± 1 °C. Il faut normalement cinq récipients au moins, renfermant chacun 10 H. azteca, *par variante de l'expérience. L'épreuve peut porter sur une seule concentration (p. ex. des échantillons non dilués de sédiment prélevé sur place) ou sur une gamme de concentrations (p. ex. sédiment enrichi de différentes concentrations de substances chimiques ou d'autre sédiment) afin de déterminer le seuil à partir duquel se manifeste l'effet. Au début de l'épreuve, l'âge des amphipodes est de deux à neuf jours.*

L'épreuve peut être effectuée avec ou sans renouvellement de l'eau surnageante. Le renouvellement de l'eau de chaque récipient doit se faire deux fois par jour, et l'eau n'est normalement pas aérée. Si on préfère ne pas renouveler l'eau, on l'aère cependant. Quelle que soit l'option retenue, les sujets de l'expérience sont nourris avec un mélange de levure, de CerophyllMC *et de nourriture pour truite (*trout chow*) [LCT]. On ajoute une suspension aqueuse de LCT dans chaque récipient, soit journellement, à raison de 1,5 mL (ce qui équivaut à environ 2,7 mg, poids sec), soit trois fois par semaine, en des journées non consécutives, à raison de 3,5 mL (environ 6,3 mg, poids sec). Le choix du régime dépend de l'objectif de l'étude et peut-être aussi des lignes directrices ou des exigences réglementaires.*

*Le rapport expose les conditions et le mode opératoire généraux et universels de la préparation de l'épreuve et de son exécution. S'y ajoutent des conditions et des modes opératoires propres à la finalité prévue de l'épreuve. Cette dernière permet de mesurer et d'évaluer la toxicité d'échantillons de sédiment, de boue ou de matière particulaire semblable, prélevés sur le terrain ou de sédiment enrichi (additionné) au laboratoire de substances ou de produits chimiques, de sédiment contaminé ou d'autres matières particulaires. Le rapport renferme également des instructions et énonce des exigences sur les installations servant à la réalisation des épreuves, sur le prélèvement des échantillons, leur manutention et leur entreposage, l'élevage d'*H. azteca, *la préparation des mélanges de sédiments ou l'addition de produits chimiques ou sédiments ainsi que sur la mise en branle des épreuves, les conditions expérimentales précises, les observations et les mesures convenables à faire, les paramètres de mesure et les méthodes de calcul de même que l'emploi de toxiques de référence.*

Foreword

*This is one of a series of **recommended methods** for measuring and assessing the aquatic biological effects of toxic substances or materials. Recommended methods are those that have been evaluated by Environment Canada (EC), and are favoured:*

- *for use in EC aquatic toxicity laboratories;*
- *for testing that is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in the absence of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as might be required in a regulatory protocol or standard reference method.*

The different types of tests included in this series were selected because of their acceptability for the needs of programs for environmental protection and management carried out by Environment Canada. These reports are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on the toxicity to aquatic life of specific test substances or materials destined for or within the aquatic environment. Depending on the biological test method chosen, substances or materials to be tested for toxicity could include samples of chemical or chemical substance, effluent, elutriate, leachate, receiving water or, where appropriate, sediment or similar particulate material.

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List of Abbreviations and Chemical Formulae

ANOVA	analysis of variance
$CaCO_3$	calcium carbonate
$CaSO_4$	calcium sulphate
$CdCl_2$	chloride
$CuSO_4$	copper sulphate
cm	centimetre(s)
CV	coefficient of variation
d	day(s)
DO	dissolved oxygen (concentration)
g	gram(s)
h	hour(s)
ICp	inhibiting concentration for a (specified) percent effect
KCl	potassium chloride
kg	kilogram(s)
L	litre(s)
LC50	median lethal concentration
LOEC	lowest-observed-effect concentration
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mS	millisiemens
MSD	minimum significant difference
mm	millimetre(s)
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NOEC	no-observed-effect concentration
O_2	oxygen
SD	standard deviation
s	second
TOEC	threshold-observed-effect concentration
TM (™)	Trade Mark
v:v	volume-to-volume
YCT	yeast, Cerophyll™, and trout chow
°C	degree(s) Celsius
µg	microgram(s)
µm	micrometre(s)
µmhos/cm	micromhos per centimetre
>	greater than
<	less than
≥	greater than or equal to

≤	less than or equal to
±	plus or minus
%	percentage or percent
‰	parts per thousand

Terminology

Note: all definitions are given in the context of the procedures in this report, and might not be appropriate in another context.

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

Might is used to express the possibility that something could exist or happen.

General Technical Terms

Acclimation is physiological adjustment to a particular level of one or more environmental factors such as temperature. The term usually refers to controlled laboratory conditions.

Compliance means in accordance with governmental permitting or regulatory requirements.

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in solution, their valence and mobility, and on the solution's temperature. Conductivity is reported as micromhos per centimetre (µmhos/cm) or as millisiemens per metre (mS/m); 1 mS/m = 10 µmhos/cm.

Flow-through describes apparatus or tests in which solutions or overlying water in culture or test chambers are/is renewed continuously by the constant inflow of a fresh solution.

Intermittent renewal describes a toxicity test in which test solutions or overlying water are/is renewed periodically during the test, usually at the beginning of each 12-h or 24-h period of testing. Synonymous terms are *batch replacement, renewed static, renewal, static renewal, static replacement,* and *semistatic*.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality, or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water quality variables, or the collection and testing of samples of sediment for toxicity.

Percentage (%) is a concentration expressed in parts per hundred. With respect to test substances, ten percent (10%) represents ten units or parts of substance diluted with sediment or water to a total of 100 parts. Depending on the test substance, concentrations can be prepared on a weight-to-weight, weight-to-volume, or volume-to-volume basis, and are expressed as the percentage of test substance in the final sediment mixture or solution.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 indicating increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Photoperiod is the duration of illumination and darkness within a 24-h day.

Pretreatment means treatment of a sediment sample, or portion thereof, before exposure of amphipods.

Salinity is the total amount of solid substance, in grams, dissolved in 1 kg of (sea)water; and is traditionally expressed as parts per thousand (‰). It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1995).

Static describes toxicity tests in which test solutions or overlying water are not renewed during the test.

Terms for Test Materials or Substances

Chemical is, in this report, any element, compound, formulation, or mixture of a substance that might be mixed with, deposited in, or found in association with sediment or water.

Clean sediment is sediment that does not contain concentrations of any substance(s) causing discernible distress to the test organisms or reducing their survival or growth during the test.

Contaminated sediment is sediment containing chemical substances at concentrations that pose a known or potential threat to environmental or human health.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no added test material or substance. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., temperature, health of test organisms, or effects due to their handling).

Control/dilution water is the water used for preparing a series of concentrations of a test chemical, or that used as overlying water in a sediment toxicity test or as control water in a *water only* test with a reference toxicant. Control/dilution water is frequently identical to the culture and test (overlying) water.

Control sediment is *clean* sediment not containing concentrations of one or more contaminants that could affect the survival, growth, or behaviour of the test organisms. Control sediment might be natural sediment from an uncontaminated site, or formulated (reconstituted) sediment. This sediment must contain no added test material or substance, and must enable acceptable (i.e., ≥80%) survival of the test organisms during the test. The use of control sediment provides a basis for interpreting data derived from toxicity tests using test sediment(s), and also provides a base sediment for spiking procedures.

Dechlorinated water is a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.

Deionized water is water that has been purified by passing it through resin columns or a reverse osmosis system, for the purpose of removing ions such as Ca^{++} and Mg^{++}.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Overlying water is water placed over sediment in a test chamber.

Pore water (also called *interstitial* water) is the water occupying space between sediment particles.

Reconstituted water is high purity deionized or glass distilled water to which reagent grade chemicals have been added. The resultant synthetic fresh water should be free from contaminants and have the desired pH, alkalinity, and hardness characteristics. Reconstituted water can also be fresh water to which commercially available dry ocean salt or brine has been added, in a quantity that provides the seawater salinity (and pH) desired for culturing organisms and for testing purposes (e.g., for a test using estuarine sediment).

Reference sediment is a field-collected sample of presumably *clean* sediment, selected for properties (e.g., particle size, compactness, total organic content) representing sediment conditions that closely match those of the sample(s) of test sediment except for the degree of chemical contaminants. It is often selected from a site uninfluenced by the source(s) of contamination but within the general vicinity of the sites where samples of test sediment are collected.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms in order to establish confidence in the toxicity data obtained for a test material or substance. In most instances, a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test material or substance is evaluated, and the precision and reliability of results obtained by the laboratory for that chemical.

Reference toxicity test is a test conducted using a reference toxicant in conjunction with a sediment toxicity test, to appraise the sensitivity of the organisms at the time the test material or substance is evaluated, and the precision and reliability of results obtained by the laboratory for that chemical. Deviations outside an established normal range indicate that the sensitivity of the test organisms, and the performance and precision of the test, are suspect. A reference toxicity test is most often performed in the absence of sediment (i.e., as a *water only* test), although it can also be conducted as a *spiked sediment* test.

Sediment is natural particulate material, which has been transported and deposited in water and usually lies below water. The term can also describe a substrate that has been experimentally prepared (formulated) using selected particulate material (e.g., sand of particular grain size, bentonite clay, etc.) and within which the test organisms can burrow.

Solid-phase sediment (also called *whole* sediment) is the intact sediment used to expose the test organisms, not a form or derivative of the sediment such as pore water or a resuspended sediment.

Spiked control sediment is clean control sediment to which a test substance or material such as a chemical, a mixture of chemicals, drilling mud, contaminated dredge spoil, sludge, or contaminated sediment has been added experimentally, and mixed thoroughly to evenly distribute the substance or material throughout the control sediment.

Spiked sediment is any sediment (clean or contaminated) to which a test substance or material such as a chemical, a mixture of chemicals, drilling mud, contaminated dredge spoil, sludge, or contaminated sediment has been added experimentally, and mixed thoroughly to evenly distribute the substance or material throughout the sediment.

Stock solution is a concentrated solution of the substance to be tested. Measured volumes of a stock solution are added to dilution water to prepare the required strengths of test solutions.

Substance is a particular kind of material having more or less uniform properties.

Test sediment is a field-collected sample of solid-phase sediment, taken from a site thought to be contaminated with one or more chemicals, and intended for use in the toxicity test with amphipods. In some instances, the term also applies to any sediment sample or mixture of spiked sediment (including control and reference sediment) used in the test.

Test water is the water placed over the layer of sediment in the test chambers, i.e., *overlying* water. It also denotes the water used to manipulate the sediment, if necessary (e.g., for preparing formulated sediment or mixtures of spiked sediment, or for wet sieving), and that used as control/dilution water for *water only* tests with reference toxicants.

Statistical and Toxicological Terms

Coefficient of Variation (CV) is the standard deviation (SD) of a set of data divided by the mean, expressed as a percentage. It is calculated as:

$$CV\ (\%) = 100\ SD \div mean.$$

Endpoint means the variable(s) (i.e., time, reaction of the organisms, etc.) that indicate(s) the termination of a test, and also means the measurement(s) or derived value(s) that characterize the results of the test (e.g., LC50, ICp, NOEC, LOEC).

ICp is the inhibiting concentration for a (specified) percentage effect. It represents a point estimate of the concentration of test material or substance that causes a designated percent impairment in a quantitative biological function such as growth. For example, an IC25 could be the concentration estimated to cause a 25% reduction in dry weight attained at the end of the test by the test organisms, relative to that in the control. This term should be used for any toxicological test that measures a continuously variable effect, such as dry weight at test end, reproduction, or respiration.

LC50 is the median lethal concentration, i.e., the concentration of substance or material in sediment (e.g., mg/kg) or water (e.g., mg/L) estimated to be lethal to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of mortalities in five or more test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 96-h LC50 for a *water only* reference toxicity test, or 14-d LC50 for a *survival-and-growth* sediment toxicity test, using *Hyalella azteca*).

Lethal means causing death by direct action. Death of amphipods is defined as the cessation of all visible signs of movement or activity indicating life (e.g., absence of a pleopod twitch).

LOEC is the lowest-observed-effect concentration. This is the lowest concentration of a test substance or material to which organisms are exposed, that causes observed and statistically significant sublethal effects on the organism. For example, the LOEC might be the lowest concentration at which the dry weight of exposed organisms at test end was significantly less than that in the control groups.

Minimum Significant Difference (*MSD*) means the difference between values for individual treatments (in this test with *H. azteca*, the difference in average weights or average mortality) that would have to exist before it could be concluded that there was a significant difference between the groups. MSD is provided by certain statistical tests including *Dunnett's multiple-range test*, a standard statistical procedure.

NOEC is the no-observed-effect concentration. This is the highest concentration of a test substance or material to which organisms are exposed, that does not cause any observed and statistically significant sublethal effects on the organism. For example, the NOEC might be the highest test concentration at which an observed variable such as dry weight at test end is not decreased significantly from weight in the control groups.

Precision refers to the closeness of repeated measurements of the same quantity to each other, i.e., the degree to which data generated from replicate measurements differ. It describes the degree of certainty around a result, or the tightness of a statistically derived endpoint such as an ICp.

Replicate refers to a single test chamber containing a prescribed number of organisms in either one concentration of the test material or substance, or in the control or reference treatment(s). In a toxicity test comprising five replicate samples of undiluted field-collected sediment taken from each of four sites (including a reference site) plus replicate samples of control sediment, 25 test chambers would be used. For each treatment (i.e., for a particular sediment-collection site or, in the case of a multi-concentration test with contaminant-spiked sediment), there would normally be a minimum of five test chambers or replicates. A replicate is an independent test unit; therefore, any transfer of organisms or test material from one replicate to another would invalidate the test.

Sublethal means detrimental to the amphipod, but below the level that directly causes death within the test period.

Sublethal effect is an adverse effect on an organism, below the level which directly causes death within the test period.

TOEC is the threshold-observed-effect concentration. It is calculated as the geometric mean of NOEC and LOEC. In some other countries, the concentration calculated in this way might be called the MATC (maximum acceptable toxicant concentration). *Chronic value* and *subchronic value* are alternative terms that have been used elsewhere and might be appropriate depending on the duration of the test.

Toxicity is the inherent potential or capacity of a substance or material to cause adverse effect(s) on living organisms. The effect(s) could be lethal or sublethal.

Toxicity test is a procedure for determining the effect of a substance or material on a group of selected organisms (e.g., *H. azteca*), under defined conditions. An aquatic toxicity test usually measures: (a) the proportions of organisms affected (*quantal*); and/or (b) the degree of effect shown (*quantitative* or *graded*), after exposure to a specific test substance or material (e.g., a sample of sediment) or mixture thereof (e.g., a chemical/sediment mixture).

Water renewal describes the renewal of the overlying water in test chambers, on a regular and timed basis (e.g., one or two times daily) throughout the test. This may be done manually or using an automated system which enables intermittent renewal of overlying water at a fixed rate.

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The report is based on existing reports (published or otherwise) that describe procedures and conditions used in the United States and Canada for culturing Hyalella azteca *and for conducting sediment toxicity tests using this species of freshwater amphipod. In particular, the specific procedures and conditions for culturing and testing* H. azteca *recommended by the United States Environmental Protection Agency (USEPA, 1994a), together with subsequent intra- and interlaboratory studies with* C. riparius *undertaken by Canadian and U.S. researchers (Milani* et al., *1996), guided the development of this biological test method. With regard to the method development and validation studies (Milani* et al., *1996), we gratefully acknowledge the participation and contribution of the following individuals and organizations: D. Milani and S. Kirby (National Water Research Institute, Environment Canada, Burlington, ON); K. Doe (Environment Canada, Moncton, NB); K. Holtze (B.A.R. Environmental Inc., Guelph, ON); A. Putt (Springborn Laboratories Inc., Wareham, MA); P. Riebel (Beak Consultants Ltd., Dorval, PQ); and G. van Aggelen (Environment Canada, North Vancouver, BC).*

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Introduction

1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to determine and monitor the toxic effects of discrete substances or complex mixtures that might be harmful to indigenous aquatic life in the environment (water and sediment). The results of toxicity tests can be used to determine the need for control of discharges, to set effluent standards, and for research and other purposes. Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Aquatic Toxicity Group (IGATG) (Appendix A) proposed a set of aquatic toxicity tests that would be broadly acceptable for use in Canada, and would measure different toxic effects using different test substances or materials (e.g., samples of chemical or chemical substance, effluent, receiving water, or sediment) and organisms representing different trophic levels and taxonomic groups.

In 1987, Environment Canada and the IGATG recommended that a consistent set of sediment testing methods be developed for routine use in preventing, appraising, remediating, and managing contaminated sediment (Sergy, 1987). At that time, Environment Canada's regional laboratories (Appendix B) began a series of studies (McLeay *et al.*, 1989; 1991; 1992; 1993; Paine and McPherson, 1991a; b; Doe and Wade, 1992; Yee *et al.*, 1992) to develop and validate a standardized biological test method for measuring the toxicity of samples of contaminated sediment. The test method would use one or more of six species of marine or estuarine amphipods common to Canadian Pacific or Atlantic coastal waters (EC, 1992a). A test for survival and growth in sediment, using the freshwater amphipod *Hyalella azteca*, was one of the sediment toxicity tests selected by the IGATG members to be standardized by Environment Canada. This would become part of a series of biological test methods prepared by Environment Canada to help meet Canadian requirements related to environmental appraisal and protection. The widespread distribution and common occurrence of *H. azteca* in association with freshwater sediment, together with its ecological importance, ease of culturing and handling during testing, rapid growth, short life cycle, sensitivity to contaminants in sediment, and extensive use in sediment toxicity tests, led to this selection.

Tests with samples of freshwater sediment and the freshwater amphipod *H. azteca* have historically been carried out by Canadian investigators using various procedures including those published by Borgmann and Munawar (1989), Borgmann *et al.* (1989), and ASTM (1991a; 1993), as well as the unpublished standard operating procedure of the National Water Research Institute (NWRI, 1992). Other notable procedures for culturing and testing *H. azteca*, which have influenced the preparation of this biological test method, include: de March, 1981; FDA, 1987; Ingersoll and Nelson, 1990; USEPA, 1991a; b; DFO, 1992; Ankley *et al.*, 1993a; Brooke *et al.*, 1993; Kubitz, 1993a; b; Norberg-King, 1992; and Smith *et al.*, 1991a; b.

In 1994, the United States Environmental Protection Agency (USEPA) published new methods for measuring the toxicity of sediment associated contaminants which include a solid-phase sediment toxicity test using *H. azteca*[1] (USEPA, 1994a). These sediment assays have been adopted as standard test methods by Committee E47 of the American Society for Testing and Materials (ASTM, 1995a). This biological test method developed by Environment Canada (EC), relies heavily on the specific procedures for culturing and testing *H. azteca* detailed in USEPA (1994a). A significant distinction, though, is that the toxicity test using *H. azteca* described in USEPA (1994a) and ASTM (1995a) is a 10-day assay for effects on amphipod survival, whereas Environment Canada's method is a 14-day *survival-and-growth* test. Additionally, this method includes two test options: (1) an intermittent-renewal test in keeping with USEPA (1994a) and ASTM (1995a), which requires twice-daily renewal of the overlying water in test chambers and normally no aeration of the overlying water; and (2) a static toxicity test in which the overlying water is not renewed (except for replacing losses due to evaporation) and is aerated continuously. Using either test option, the animals are fed a standardized mixture of yeast, Cerophyll™, and trout chow (YCT), either as a daily inoculum of 1.5 mL (equivalent to ~2.7 mg dry weight), or three times per week on nonconsecutive days using an inoculum of 3.5 mL (~6.3 mg dry weight).

Universal procedures for preparing and conducting sediment toxicity tests using *H. azteca* are described in this report. Also presented are specific conditions and procedures which are required or recommended when using the test for evaluating different types of substances or materials (e.g., samples of field-collected sediment or particulate waste, or samples of one or more chemicals or chemical substances experimentally mixed into or placed in contact with natural or formulated sediment).

The flowchart in Figure 1 gives a general picture of the universal topics covered herein, and lists topics specific to testing samples of field-collected sediment, similar particulate waste (e.g., sludge, drilling mud, or dredged material), or sediment spiked experimentally with chemical(s), contaminated sediment, or particulate waste. This biological test method has been developed following a review of variations in specific culturing and test procedures indicated in existing Canadian and United States methodology documents[2] that describe how to prepare and

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[1] USEPA (1994a) was prepared by members of the United States Freshwater Sediment Toxicity Assessment Committee, and reflects a consensus opinion of U.S. and Canadian researchers actively engaged in sediment toxicity tests using *H. azteca* (Ingersoll, 1992; Norberg-King, 1992; Burton and Ingersoll, 1994; Ingersoll *et al.*, 1995; Burton *et al.*, 1996).

[2] Documents used in preparing listings of procedural specific variations (see Appendices C, D, and E) include published "how-to" references, unpublished Standard Operating Procedures of governmental testing facilities, and draft reports. Citations of source documents are listed in these appendices by originating agency, rather than by author(s), although the authors and formal citations are identified in the appendices.

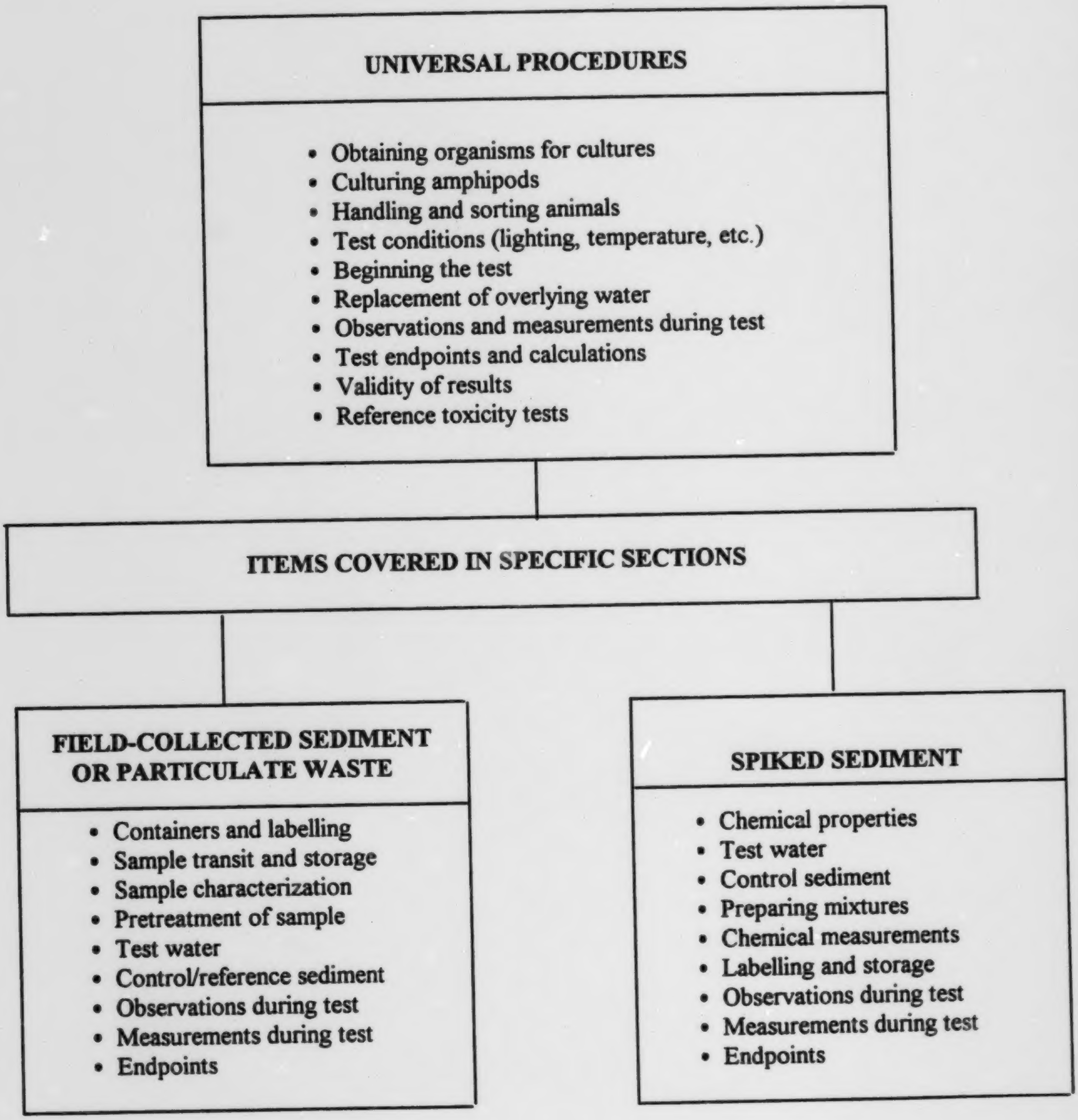
UNIVERSAL PROCEDURES
• Obtaining organisms for cultures
• Culturing amphipods
• Handling and sorting animals
• Test conditions (lighting, temperature, etc.)
• Beginning the test
• Replacement of overlying water
• Observations and measurements during test
• Test endpoints and calculations
• Validity of results
• Reference toxicity tests
ITEMS COVERED IN SPECIFIC SECTIONS
FIELD-COLLECTED SEDIMENT OR PARTICULATE WASTE
• Containers and labelling
• Sample transit and storage
• Sample characterization
• Pretreatment of sample
• Test water
• Control/reference sediment
• Observations during test
• Measurements during test
• Endpoints
SPIKED SEDIMENT
• Chemical properties
• Test water
• Control sediment
• Preparing mixtures
• Chemical measurements
• Labelling and storage
• Observations during test
• Measurements during test
• Endpoints

Figure 1 **Considerations for Preparing and Performing Toxicity Tests Using *Hyalella azteca* and Various Types of Test Materials or Substances**

conduct sediment toxicity tests using the freshwater amphipod *H. azteca*. A summary of existing or past procedural variations for culturing this species and for harvesting young for use in toxicity tests is found in Appendix C. A summary of variations in existing or past procedures for conducting growth and/or survival tests for sediment toxicity using *H. azteca* is found in Appendix D. Appendix E provides a summary of interlaboratory variations in conditions and procedures for undertaking reference toxicity tests with *H. azteca*.

The biological endpoints for this test method are mean percent survival and mean dry weight (as an indication of growth) at the end of the 14-day test. The test method is intended for use in evaluating the toxicity of samples of:

(1) field-collected freshwater sediment;

(2) industrial or municipal sludge and similar particulate wastes that might affect the freshwater environment; and

(3) mixtures of one or more chemicals or chemical substances within or overlying freshwater sediment.

A 10-day test method for toxic effects of these materials on survival and growth of larval freshwater midges (*Chironomus tentans* or *C. riparius*) has also been developed by Environment Canada (1997a), and may be used in conjunction with or as an alternative to this test. The present test method may also be used to measure and appraise the toxicity of contaminants in estuarine sediment, or of chemical/sediment mixtures where the salinity of the overlying and/or pore water does not exceed 15 ‰ (Nebeker and Miller, 1988; USEPA, 1994a). Environment Canada's biological test method using one or more recommended species of estuarine or marine amphipods common to Canada's Atlantic or Pacific coastal waters (EC, 1992a) is normally used for measuring and evaluating the toxicity of contaminants in estuarine or marine sediment.

In formulating these procedures, an attempt has been made to balance scientific, practical, and cost considerations, and to ensure that the results will be sufficiently precise for most situations in which they will be applied. It is assumed that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions that might be required in a regulatory protocol are not provided in this report, although it is intended as a guidance document useful for that and other applications.

For guidance on the implementation of this and other biological test methods, and on the interpretation and application of the endpoint data, the reader should consult Environment Canada (1997b).

1.2 Identification, Distribution, and Life History

H. azteca (Saussure) is an epibenthic, detritus-feeding, sediment-burrowing, freshwater amphipod. The distinguishing features of this small (male to 8 mm, female to 6 mm) crustacean species are described and illustrated in Appendix F.

H. azteca resides in temperate lakes, ponds, and slow-flowing streams, in close association with the surficial 1 or 2 cm of sediment. The species has achieved densities of >10 000/m^2 in preferred habitats and can also be found in lower numbers in sloughs, ditches, springs, rivers, and marshes (USEPA, 1994a). The species is widely distributed on the North American continent, and has been found in surficial sediments of lakes from Guatemala and the Caribbean Islands to Inuvik, Northwest Territories (de March, 1981). Information on the known distribution of *H. azteca* in Canadian waters, and its habitat, is included in Appendix F.

Amphipods are an abundant component of benthic communities in North American freshwater, estuarine, and marine environments. Freshwater amphipods including *H. azteca* are an important source of food for many species of fish, waterfowl, wading birds, salamanders, and larger invertebrates (de March, 1981). *H. azteca* is reported to selectively ingest bacteria and algae that adhere to sediment particles <65 µm (Hargrave, 1970). The animal reproduces sexually and the eggs and live young are carried in a brood pouch on the female's ventral surface. Immediately before mating, the female molts and releases its offspring from the previous mating. Depending on the size and condition of the female, 1 to 50 offspring can be produced and released at each molt. At 25°C, *H. azteca* reaches sexual maturity in about 28 to 33 days (de March, 1981; USEPA, 1991a; b). For further details on the life history of this species see Appendix F.

1.3 Historical Use in Toxicity Tests

Burton (1991) provides an excellent review of the various methods used historically for measuring the toxicity of freshwater sediments, including toxicity tests with *H. azteca* and other species of freshwater amphipods. Nebeker *et al.* (1984) first recommended that *H. azteca* be used in partial life-cycle tests to measure the toxicity of contaminated freshwater sediment. Nebeker and Miller (1988) demonstrated that this species of amphipod will survive and reproduce in toxicity tests with sediments from estuarine sites when the sediment in the test chambers is overlain by fresh water. Subsequent studies have demonstrated that *H. azteca* can be cultured in water with a salinity of up to 15‰, then used in toxicity tests associated with estuarine discharges (Ingersoll *et al.*, 1992) or contaminated estuarine sediments (McGee *et al.*, 1993). The USEPA and the United States Army Corps of Engineers (USACE) have recommended that *H. azteca* be used for evaluating dredged material proposed for discharge in inland and coastal waters (USEPA/USACE, 1994). Ingersoll *et al.* (1995) reviewed methods and applications for sediment toxicity tests using *H. azteca*.

H. azteca has been used in *water only* acute and chronic toxicity tests with various chemicals (FDA, 1987; Borgmann and Munawar, 1989; Borgmann *et al.*, 1989; 1990; 1991; 1993; Borgmann, 1994; Schubauer-Berigan *et al.*, 1993; Hoke *et al.*, 1995; Phipps *et al.*, 1995). Biological endpoints for these tests have included survival, growth, and reproductive success in partial or full (12 to 14 weeks at 25°C) life-cycle studies. *Water only* tests for bioaccumulation of specific chemicals have also been conducted using *H. azteca* (Borgmann *et al.*, 1990; 1991; 1993). The toxicity and bioaccumulation of chemicals added to sediment (*spiked sediment* tests) have been studied by several researchers using *H. azteca* (Landrum and Scavia, 1983; Cairns *et al.*, 1984; Nebeker *et al.*, 1986;

1989; Smith *et al.*, 1992a; Suedel *et al.*, 1993a; b; Kubitz *et al.*, 1995; Milani *et al.*, 1996; Whiteman *et al.*, 1996).

Many investigators have successfully used *H. azteca* to appraise the toxicity of whole (*solid phase*) samples of freshwater sediment (e.g., Nebeker *et al.*, 1984; Borgmann and Munawar, 1989; Burton *et al.*, 1989; Ingersoll and Nelson, 1990; Ankley *et al.*, 1993a; b; Borgmann and Norwood, 1993; Kubitz, 1993a; Kubitz *et al.*, 1993; Sibley *et al.*, 1993; West *et al.*, 1993; Burton and Ingersoll, 1994; Burton *et al.*, 1996; Kemble *et al.*, 1994; Pastorok *et al.*, 1994; Becker *et al.*, 1995; Ingersoll *et al.*, 1995; Kubitz *et al.*, 1995; 1996; Reynoldson *et al.*, 1995; Milani *et al.*, 1996). Biological endpoints for these tests, which can be used to evaluate the spatial and temporal variability in toxicity of samples of field-collected sediment, are typically mean percent survival and mean growth (length or weight) at the end of the test. Toxicity tests using aqueous extracts (pore water and or elutriates) of freshwater sediments have also been conducted using *H. azteca* (Burton *et al.*, 1989; Ankley *et al.*, 1991; Schubauer-Berigan and Ankley, 1991; Sibley *et al.*, 1993).

Results of laboratory sediment toxicity tests using *H. azteca* have recently been examined to assess their worth in identifying sites where natural populations of benthic organisms are affected by toxic sediment contaminants (Becker and Bigham, 1993; Burton and Ingersoll, 1994; Canfield *et al.*, 1994; Schlekat *et al.*, 1994). Such *field validation* studies typically integrate the findings of the laboratory toxicity tests with concurrent chemical analyses of sediment samples and field surveys of the diversity and abundance of benthic communities, using a Sediment Quality Triad (Chapman *et al.*, 1986; 1987; 1991) or similar approach. To date, integrated laboratory and field studies which include sediment toxicity tests using *H. azteca* or other species of amphipods have indicated that these tests can provide reliable evidence of biologically adverse contamination of sediment in the field (Swartz *et al.*, 1982; 1985a; 1986; 1994; Becker *et al.*, 1990; Canfield *et al.*, 1994; USEPA, 1994a; Day *et al.*, 1995a).

1.4 Laboratory Tolerance and Relative Sensitivity

A number of studies have examined the tolerance of *H. azteca* to certain natural environmental variables under laboratory conditions. Effects of temperature on the tolerance, behaviour, and reproductive biology of this animal are summarized in de March (1981). The natural range of temperatures tolerated by *H. azteca* is 0 to 33°C. Generally, maximum numbers of young are produced between 26 and 28°C, whereas temperatures of 33 to 37°C are lethal. Temperatures of 0 to 10°C cause complete immobility; temperatures of 10 to 18°C delay maturation, result in a low rate of reproduction, and produce large adults; and temperatures ≥20°C decrease maturation time, increase the rate of reproduction, and produce small adults (de March, 1981).

Reproduction of *H. azteca* in the laboratory is successful and continuous if a photoperiod of at least 16 hours of light per day (≥16L:8D) is used. Shorter daylight hours (≤12L:12D) can result in a reproductive resting stage (de March, 1977). *H. azteca* has been cultured successfully using broad-spectrum fluorescent lighting with an intensity of about 500 to 1000 lux (Ingersoll and Nelson, 1990; Ankley *et al.*, 1991; USEPA, 1994a). Covering culture jars with aluminum foil did not affect survival, but reduced growth rates and eliminated reproduction (Borgmann *et al.*, 1989).

H. azteca can survive exposure to low levels of dissolved oxygen for extended periods. de March (1981) summarized studies indicating that this species could survive in stagnant water, and cited two independent investigations which reported a 48-h LC50 of 0.7 mg O_2/L for this species. Nebeker *et al.* (1992) also found that this animal could survive acute or prolonged exposure to low dissolved oxygen levels; both 96-h and 30-d LC50s were <0.3 mg O_2/L. However, growth and reproduction (mean number of young) were both reduced after 30 days' exposure to water with ≤1.2 mg O_2/L. The effects of higher (but below saturation values) concentrations of dissolved oxygen on growth and reproduction of *H. azteca* were not investigated by Nebeker *et al.* (1992).

Little information is available on how pH affects the survival of *H. azteca*. de March (1979) reported that survival of this species was optimum at pH values of 6 to 8, and that pH values ranging from 4 to 5 resulted in gradual mortalities. It is not known if there is any information on the effects of fresh water with differing pH values on the growth rates or reproductive success of this amphipod.

There is little definitive information on the influence of water hardness or alkalinity on the wellbeing of *H. azteca*. The USEPA (1991b) observed that the reproductive success of this species was often poor when cultured in reconstituted water adjusted to low hardness values using conventional recipes (e.g., those in USEPA, 1985a; b; 1991c). However, this problem might have been due to a chemical imbalance of the ions in solution for this species, rather than adverse hardness *per se*; and a recipe for preparing reconstituted water with a hardness of 90 to 100 mg $CaCO_3$/L has yielded better (although not universal) success (USEPA, 1994a; see Section 2.3.4). Further research is required to determine the ranges of water hardness and alkalinity that are suitable for culturing and testing *H. azteca*.

H. azteca has been shown to be euryhaline, and the species has been successfully cultured and/or tested using estuarine water and estuarine sediment. In tests using organisms acclimated to fresh water, Nebeker and Miller (1988) reported that, depending on age of the test organisms (i.e., young adults or mature adults), 10-d LC50s for *H. azteca* exposed to various salinity concentrations ranged from 19 to 24‰ and 24-h LC50s for mature adults ranged from 16 to 19‰. For organisms acclimated to fresh water, inhibition of reproductive success (i.e., number of young produced) was evident at salinities of 10.4‰ and higher (Nebeker and Miller, 1988). Presumably, the salinity tolerance of this species might be greater if the animals are acclimated to estuarine water before testing. de March (1981) noted that *H. azteca* can survive a salinity as high as 30‰ if acclimated gradually. Other studies have demonstrated that *H. azteca* can be cultured successfully in water with a salinity of 15‰ or less (McGee *et al.*, 1993; USEPA, 1994a).

The influence of natural physicochemical properties of sediments on the performance of *H. azteca* in sediment toxicity tests has been examined. Ingersoll and Nelson (1990) found that this species has an extremely wide tolerance of sediment grain size. In long-term exposures to clean sediments ranging from >90% silt- and clay-sized particles to 100% sand-sized particles, no detrimental effects on either survival or growth were noted. Similarly, Ankley *et al.* (1994) conducted 10-day *H. azteca* sediment assays using 50 uncontaminated samples of lake sediment with particle sizes ranging from 95% clay to 100% sand, and organic carbon content from 0.3 to 8.1%. These researchers found no correlation between amphipod survival rates and sediment characteristics including particle size, organic carbon content, or mineralogical composition, provided the animals were fed during the tests. In 10-day survival tests with laboratory-formulated or clean field-collected sediments, Suedel and Rodgers (1994a) determined that *H. azteca* was tolerant of all of the sediment particle size distributions (0 to 100% sand, 0 to 100% silt, and 0 to 60% clay) and ranges of organic carbon content (0.1 to 8.0%) examined. In 48-h tests with sediment spiked using a range of concentrations of alkylbenzene sulphonate, Cano *et al.* (1996) found that enriching the sediment with peat moss increased the acute lethal tolerance of *H. azteca* to this surfactant when total organic carbon content was ≥1.5%.

The sensitivity of *H. azteca* to sediments or chemicals, relative to that of other freshwater species commonly used in toxicity tests, has been evaluated in a number of studies. Reviews of comparative toxicity data indicate that *H. azteca* is one of the most sensitive freshwater species (Burton, 1991; USEPA, 1994a). Acute lethality *water only* tests with a number of industrial effluents indicated that the sensitivity of *H. azteca* was similar to that of rainbow trout, *Oncorhynchus mykiss* (Maciorowski, 1975). Similarly, results for comparative 96-h (*H. azteca*) or 48-h (*Ceriodaphnia dubia*) *water only* tests using potassium chloride (KCl) showed that the acute lethal tolerance of *H. azteca* and the freshwater daphnid *C. dubia* to this reference toxicant was similar (Smith *et al.*, 1991b). In comparative acute lethality tests with sediment pore water or elutriate, *H. azteca* was either as, or slightly more, sensitive than *C. dubia* or larval fathead minnows (*Pimephales promelas*), with the oligochaete *Lumbriculus variegatus* being the least sensitive (Ankley *et al.* (1991). Chronic *water only* tests with cadmium and pentachlorophenol, using *H. azteca*, another amphipod (*Gammarus fasciatus*), or *Daphnia magna* as test organisms, showed that the sensitivities of the two amphipod species were similar and that each species was as, or more, sensitive than *D. magna* (Borgmann *et al.*, 1989). Comparative 10-day *water only* and *spiked sediment* tests with fluoranthene, using *H. azteca*, larval freshwater midges (*Chironomus tentans*), and *D. magna*, showed that *H. azteca* and *C. tentans* were twice as sensitive as *D. magna* in the water only tests, whereas *H. azteca* was as or more sensitive than the other two species in the spiked sediment tests (Suedel *et al.*, 1993a). In comparative *water only* LC50s involving *H. azteca, C. tentans,* and *L. variegatus* exposed separately to each of five metals and five pesticides, Phipps *et al.* (1995) found that no one species was consistently most sensitive to all toxicants although *H. azteca* was the species most sensitive to all five metals. Additionally, these investigators compared the LC50s derived for *H. azteca* to published toxicity values for other aquatic species exposed to the same chemicals, and noted that *H. azteca* was frequently amongst the most sensitive species (Phipps *et al.*, 1995). Comparative tests by Kubitz *et al.* (1995), using copper-spiked sediment or samples of field collected sediment, 48-h porewater tests for survival of *D. magna* or *Ceriodaphnia dubia*, 1-h tests for enzyme inhibition using *D. magna*, and 14-day sediment *survival-and-growth* tests with *H. azteca*, indicated that both the growth (i.e., dry weight) endpoint using *H. azteca* and the enzyme inhibition endpoint using *D. magna* were more sensitive than any of the survival endpoints.

In comparative 10-day *whole sediment* tests with field-collected sediment, West *et al.* (1993) found that, of the species compared (i.e., *H. azteca*, *C. tentans*, and *L. variegatus*), *H. azteca* was the most sensitive. As cited in USEPA (1994a), Kemble *et al.* (1994) compared the sensitivity of *H. azteca*, *Chironomus riparius*, *D. magna*, and rainbow trout to samples of metal-contaminated sediment. Using length, sexual maturation, and survival as endpoints in 28-day tests with *H. azteca*, results showed that *H. azteca* was the most sensitive of the four species tested (and that length was the most sensitive endpoint). In a separate study of contaminated sediment from the Great Lakes, *H. azteca* was amongst the most sensitive and discriminatory of 24 organisms tested (Burton and Ingersoll, 1994; USEPA, 1994a). The results of studies by Smith *et al.* (1993) indicate that larval fathead minnows (7-day test) might be more sensitive than *H. azteca* to certain natural sediments contaminated with metals and metalloids (selenium).

Test Organisms

2.1 Species and Life Stage

The freshwater amphipod *Hyalella azteca* is used in this biological test method. This crustacean species is an epibenthic, sediment-burrowing detritivore that lives in close contact with freshwater sediments. Species identification should be confirmed and documented by qualified personnel experienced in identifying amphipods, using the distinguishing taxonomic features described and illustrated in Appendix F.

Juvenile *H. azteca* that have been cultured in the laboratory and are 2- to 9-days old must be used for this biological test method. For greater standardization and reduced variability of growth measurements, it is desirable although not always practical to use animals that are more similar in age (see Section 2.3.10).

2.2 Source and Acclimation

All amphipods used in a test must be derived from the same population. Sources of animals required to establish cultures (see Section 2.3) may be government or private laboratories, which are culturing *H. azteca* for sediment toxicity tests, or a commercial biological supplier.[3] Breeding stock can be acquired from the following Canadian sources:

Toxicology Laboratory
Environmental Quality Section
Environmental Science Centre
Environment Canada
P.O. Box 23005
Moncton, NB, E1A 6S8

Aquatic Toxicology Section
Pacific Environmental Science Centre
Environment Canada
2645 Dollarton Highway
North Vancouver, BC, V7H 1V2

Persons wishing United States sources for test organisms should refer to USEPA (1994a; Table 10.1) for contacts.

Breeding stock should be transported to the laboratory using the source of water in which the organisms have been reared. Water used for transporting animals should be well oxygenated (90 to 100% saturated) before shipment, and suitable substrate should be provided (Section 2.3). Shipping containers should be insulated to minimize changes in water temperature during transit. Live organisms should be transported quickly to ensure prompt delivery (i.e., within 24 h) .

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[3] Investigators might be concerned with the effects of excessive inbreeding of laboratory cultures, or might wish to use progeny reproduced from organisms that occupied a particular locale. Accordingly, cultures may also be established using wild populations. If animals are obtained from a wild population, their taxonomy should be confirmed and they should be cultured through several generations and evaluated for sensitivity to reference toxicant(s) before the progeny are used in toxicity tests. Obtaining wild populations of organisms for testing should be avoided unless the ability of the wild population to cross-breed with existing laboratory populations has been demonstrated (USEPA, 1994a).

Excessive crowding of animals during shipment should be avoided to minimize stress and prevent oxygen deficiency in transit.

Upon arrival at the laboratory, organisms may be held in the water used in transit while temperature adjustments are made, or they may be transferred to well-oxygenated culture water adjusted to the temperature of the water in the shipping container. Gradual exposure of organisms to culture water is recommended in instances where there is a marked difference in quality (e.g., hardness, pH, conductivity) from that to which they were previously acclimated. Guidance given in Section 4.1 for acclimating organisms to test water might also be followed here when transferring amphipods from another source to culture water.

Water temperature should be adjusted gradually to the temperature specified for culturing (23°C; Section 2.3), at a rate not exceeding 2°C/day (USEPA, 1994a). During this acclimation period, water used to hold the breeding stock should be aerated gently. Other conditions during this interim holding period for acclimation of breeding stock to laboratory conditions should be as similar as possible to those used for maintaining cultures (Section 2.3).

2.3 Culturing

2.3.1 General

General guidance and recommendations for culturing *H. azteca* in preparation for sediment toxicity tests are provided here. In keeping with the premise "What might work well for one laboratory might not work as well for another laboratory" (USEPA, 1994a), explicit directions regarding many aspects of culturing, including the choice of water-renewal conditions, substrate for amphipods, and food type and ration, are left to the discretion and experience of laboratory personnel. Performance-based criteria[4] are used to evaluate the suitability of the cultured organisms for tests, and the acceptability of the test results. To be suitable for use in tests, cultures must have low mortalities, and the cultured organisms must appear healthy, exhibit normal feeding and other behaviour, and be of an age between 2- and 9-d old when a test is started. The acceptability of the culture should also be demonstrated by concurrent or ongoing tests using one or more reference toxicants (Section 4.8). If a batch of organisms fails to meet these criteria, it should be discarded.

It is the responsibility of the laboratory to demonstrate its ability to obtain consistent, precise results using one or more reference toxicants, when initially setting up to perform sediment toxicity tests with *H. azteca*. For this purpose, intralaboratory precision, expressed as a coefficient of variation for the respective LC50 data, should be determined by performing five or more tests with different batches of test organisms from the same source, using the same reference toxicant and identical procedures and conditions for each test (see Section 4.8) (USEPA, 1994a). The laboratory should also confirm its test precision at this time by conducting five or more 14-day *survival-and-growth* tests using control sediment and different batches of test organisms (USEPA, 1994a). The conditions and procedures used to perform these initial tests with control sediment should be identical and according to Section 4.

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[4] Performance-based criteria include those related to the survival and condition of cultured animals intended for use in the test (Section 2.3.11); as well as the criteria that must be met by control organisms for a test to be valid (Section 4.2), and those related to the performance of groups of animals in reference toxicity tests (Section 4.8).

When routinely performing sediment toxicity tests with *H. azteca*, reference toxicity tests should be conducted monthly with the laboratory's cultures, using the conditions and procedures outlined in Section 4.8. If this monthly routine is not followed, the performance of individuals from the culture used to start a sediment toxicity test should be evaluated in a reference toxicity test conducted concurrently. Additionally, the performance of any cultures that have been recently established using new breeding stock (Section 2.2) should be checked with a reference toxicity test, and the results determined to be acceptable (see Sections 2.3.11 and 4.8) before these cultures are used to provide test organisms.

Cultures should be observed on a frequent and routine basis (e.g., daily or, as a minimum, three times per week on nonconsecutive days). The estimated number of surviving adults and the production of young in each culture chamber, dates of culture renewals, numbers and age classes of transferred individuals, daily feedings, water quality measurements, etc. should be documented (see Section 7 *Reporting Requirements*).

A summary of the various conditions and procedures that have been used by government laboratories for culturing *H. azteca* is provided in Appendix C. These procedural specifics have presumably worked well in producing test organisms and, unless indicated otherwise in this report, provide useful guidance which may be applied here. A checklist of recommended conditions and procedures for culturing *H. azteca* to generate offspring for use in sediment toxicity tests is given in Table 1.

2.3.2 Facilities and Apparatus

H. azteca must be cultured in a controlled temperature laboratory facility. Equipment for temperature control (i.e., incubator, recirculating water bath, or constant temperature room) must be adequate to maintain the temperature within the required limits (Section 2.3.5). The culturing area must be isolated from any testing, sample storage or sample preparation areas, to avoid contamination from these sources. It must be designed and constructed to prevent contamination of cultures (e.g., elimination of copper or galvanized piping or fixtures that could drip metal-contaminated condensates). The air supply to this area should be designed and operated to prevent entry or recirculation of air from the testing facility or from other portions of the laboratory where contaminants are present.

All equipment, containers, and accessories that might contact the organisms or water within the culturing facility must be clean, rinsed as appropriate, and made of nontoxic materials (e.g., glass, Teflon™, type 316 stainless steel, nylon, Nalgene™, porcelain, polyethylene, polypropylene, fibreglass). Toxic materials including copper, zinc, brass, galvanized metal, lead, and natural rubber must not come in contact with this apparatus and equipment, or the culture water. Online compressed air to the culturing facility

Table 1 **Checklist of Recommended Conditions and Procedures for Culturing *Hyalella azteca* for Use in Sediment Toxicity Tests**[5]

Source of amphipods	–	existing government, private, or commercial culture; all animals in a test from the same source; species identification confirmed
Acclimation	–	gradually (≤2°C/day) for temperature differences upon arrival
Water source	–	uncontaminated ground, surface, reconstituted, or, if necessary, dechlorinated municipal tap water; reconstituted or natural seawater with salinity ≤15 ‰ for special needs
Water quality	–	temperature monitored daily; dissolved oxygen monitored at least weekly; pH, hardness, alkalinity, and ammonia measured during 24-h period preceding start of test
Water renewal	–	intermittent-renewal or continuous-flow; ≥1 volume addition/d recommended, 25 to 30%/week (minimum) unless water is recirculated through a filtration system
Temperature	–	23 ± 1°C as daily average, and 23 ± 3°C as instantaneous
Aeration/oxygen	–	aerate gently; maintain dissolved oxygen at ≥80% saturation
Lighting	–	500 to 1000 lux adjacent to the water surface; overhead full-spectrum tubes (fluorescent or equivalent, with a broad-spectrum wavelength); photoperiod 16-h light:8-h dark
Substrate	–	medicinal gauze bandage; other choices (e.g., see Appendix C.5) allowed
Feeding	–	various types, quantities, and rates allowed
Age for test	–	2- to 9-day old at start of test
Health criteria	–	discard batch of organisms intended for use in a test if >20% of young amphipods die or appear stressed during the 48-h period before the test

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[5] Conditions and procedures listed apply primarily to known-age cultures (Section 2.3.10), which are commonly maintained in 1- to 2-L beakers or jars, and do not necessarily apply to large or mixed-age stock cultures.

should be filtered as necessary to ensure that it is free of oil and fumes.

2.3.3 Lighting

Overhead full-spectrum lights (fluorescent or equivalent) should illuminate the cultures. The photoperiod should be regulated at 16-h light and 8-h dark, and the light intensity adjacent to the water surface in the culture chambers should range within 500 to 1000 lux (USEPA, 1994a).

2.3.4 Culture Water

Sources of water for culturing *H. azteca* may be an uncontaminated supply of groundwater, surface water, or reconstituted water. Culture water may also be prepared by diluting natural water with a high purity distilled or deionized water until a desired hardness is achieved. Acceptable water must allow satisfactory survival, growth, and reproduction of this species.

For certain site-specific investigations, the experimental design might require use of water taken from the site where sediment is collected. If this or other surface water is used, it should be filtered through a fine-mesh net (e.g., 30 µm) to remove potential predators or competitors. Water that might be contaminated with pathogens may be sterilized by passing it through an ultraviolet sterilizer.

Dechlorinated water is not recommended for use as culture or test water, since its quality is often variable and it could contain unacceptably high concentrations of chlorine, chloramines, fluoride, copper, lead, zinc, or other contaminants. Notwithstanding, certain laboratories routinely use dechlorinated municipal water for culturing *H. azteca* and as test water with no apparent problems. If municipal drinking water is used, effective dechlorination[6] must remove any harmful concentration of residual chlorine or chloramines.[7]

If reconstituted fresh water is used for culturing *H. azteca*, the recipe developed by the USEPA laboratory in Cincinnati, OH (Smith *et al.*, 1992b) might prove suitable. The following recipe, which provides reconstituted water with a hardness of 90 to 100 mg $CaCO_3$/L, is taken from USEPA (1994a). It has a higher proportion of chloride to sulphate compared to other recipes commonly used (e.g., USEPA, 1985a; b; 1991c; 1993; EC, 1992b), and in some but not all laboratories this recipe has been found to be suitable for culturing *H. azteca*.[8] To prepare 100 L of reconstituted fresh water, use the reagent grade chemicals

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[6] Vigorous aeration of the water can be applied to strip out a portion of any residual chlorine gas. This could be followed by use of activated carbon (bone charcoal) filters and perhaps subsequent ultraviolet radiation (Armstrong and Scott, 1974) for removing most of the residual chloramine and other chlorinated organic compounds. Aging the water in an aerated holding tank for 1 or 2 days might be of further benefit.

[7] The target value for total residual chlorine, recommended for the protection of freshwater aquatic life, is ≤0.002 mg/L (CCREM, 1987). Values greater than 0.002 mg/L might risk interaction of chlorine/chloramine toxicity with the contaminant(s) being tested. The limit of detection for the analytical technique used to measure residual chlorine or chloramine in the treated supply of dechlorinated water must be low enough to assure that residual chlorine is ≤0.002 mg/L.

[8] Some laboratories have experienced only marginal success in culturing *H. azteca* using reconstituted water, and prefer natural well or surface water for this purpose (G.A. Burton, Jr., Wright State Univ., Dayton, OH, personal communication, 1994).

(anhydrous salts) as follows (USEPA, 1994a):

1. Place about 75 L of high-purity deionized water in a clean (see Section 3.1) container.

2. Add 5 g of calcium sulphate ($CaSO_4$) and 5 g of calcium chloride ($CaCl_2$) to a 2-L aliquot of deionized water and mix (e.g., on a stir plate) for 30 minutes or until the salts dissolve.

3. Add 3 g of magnesium sulphate ($MgSO_4$), 9.6 g sodium bicarbonate ($NaHCO_3$), and 0.4 g potassium chloride (KCl) to a second 2-L aliquot of deionized water and mix on a stir plate for 30 minutes.

4. Pour the two, 2-L aliquots containing the dissolved salts into the 75 L of deionized water and fill the carboy to 100 L with deionized water.

5. Aerate the mixture for at least 24 h before use.

6. The water quality of the reconstituted water should be approximately the following: hardness, 90 to 100 mg/L as calcium carbonate ($CaCO_3$); alkalinity, 50 to 70 mg/L as $CaCO_3$; conductivity, 330 to 360 µS/cm; and pH, 7.8 to 8.2.

The reconstituted water should be aerated before use to adjust the dissolved oxygen to an acceptable range (see Section 2.3.6) and to stabilize pH. Conductivity, pH, hardness, dissolved oxygen, and alkalinity should be measured in each batch of reconstituted water (USEPA, 1994a). The USEPA (1991c) recommends using a batch of reconstituted water for two weeks but no longer than four weeks.

Natural or reconstituted seawater with a salinity of ≤15 ppt may be used for culturing *H. azteca* (USEPA, 1994a). Reconstituted seawater is prepared by adding hypersaline brine, an acceptable formulation of reagent-grade salts, or commercially-available dry ocean salts (e.g., Instant Ocean^{TM}) to deionized or distilled water or a suitable uncontaminated fresh water, in a quantity sufficient to provide the desired salinity (EC, 1992a; 1997c; USEPA, 1994b).

The characteristics of the water used within a laboratory for culturing *H. azteca* should be reasonably uniform, in order to improve the likelihood of intralaboratory culturing success and to minimize variations in condition and development of cultured organisms. According to USEPA (1994a), a natural water is considered to be of uniform quality if monthly ranges of the hardness, alkalinity, and specific conductance are less than 10% of their respective averages, and if the monthly range of pH is less than 0.4.

The quality of water in culture chambers should be monitored and recorded routinely. Water temperature should be measured daily, and dissolved oxygen measured at least weekly. Culture water hardness, alkalinity, pH, and ammonia should be measured as frequently as necessary to document water quality. It is recommended that these variables be measured at least quarterly, as well as on the day before the start of a test (USEPA, 1994a).

Water used for culturing *H. azteca* should be analyzed for nitrite, suspended solids, total dissolved gases, metals, pesticides, and any other contaminants of concern, as frequently as necessary to document water quality (e.g., quarterly). For each analytical method used, the detection limit should be appreciably (e.g., 3 to 10 times) below either (a) the concentration in the water, or (b) the lowest concentration that has been shown to adversely affect the survival, growth, or reproduction of *H. azteca* or other sensitive freshwater animals (EC, 1992b).

The water within culture chambers should be renewed routinely.[9] This may be accomplished manually, or automatically using suitable apparatus and techniques for continuous or intermittent renewal. A water renewal rate equivalent to ≥1 volume addition/day has been recommended (USEPA, 1994a), although such a frequent rate of exchange is likely unnecessary. A volume addition of 25 to 30% per week is the minimum exchange allowable (NWRI, 1992) unless water is re-circulated through commercial (aquarium supply) filters.

2.3.5 Temperature

The temperature of the water in culture chambers containing *H. azteca* should be 23 ± 1°C, as a daily average (Table 1). Additionally, the instantaneous temperature of the culture water should be 23 ± 3°C.

2.3.6 Dissolved Oxygen

Water to be used for cultures should be aerated vigorously just before use, to ensure adequate oxygen content and to prevent supersaturation with gases. Dissolved oxygen (DO) should be measured at this time to confirm that a satisfactory value has been obtained (e.g., 90 to 100% saturation).

Cultures should be aerated gently (e.g., 1 bubble/s for each litre of water; Brooke *et al.*, 1993) using filtered, oil-free compressed air. Air to cultures should be dispensed through disposable airline tubing and disposable glass or plastic pipettes or, for large volume cultures, aquarium supply airstones. To ensure that dissolved oxygen is adequate to sustain optimum survival and growth of amphipods, it is recommended that DO in cultures be maintained at 80 to 100% saturation.

2.3.7 Culturing Substrate

Various types of substrate have been used successfully for culturing *H. azteca* (see Appendix C.5); the choice is left to the discretion and experience of laboratory personnel. Presoaked medicinal cotton gauze strips (e.g., 5 × 10 cm or 2.5 × 2.5 cm, depending on the size of the culture chamber) are frequently used and are recommended as a suitable substrate (Borgmann *et al.*, 1989; DFO, 1992). The USEPA (1994a) recommends soaking cotton gauze in water for 24 h before use, and replacing the gauze weekly. Other materials including Nitex™ nylon mesh, plastic mesh, or shredded paper towels (Appendix C.5) may also be used.

2.3.8 Food and Feeding

Various types of food and feeding regimes have been used for culturing *H. azteca* (see Appendix C.6). Success in culturing this species has been achieved using a single ration diet such as commercial fish food flakes or rabbit chow (DFO, 1992; Ingersoll and Nelson, 1990; NWRI, 1992; Milani *et al.*, 1996), as well as a mixed diet such as filamentous algae, yeast, Cerophyll™ and trout chow (USEPA, 1991b; Brooke *et al.*, 1993). The USEPA (1994a) recommends feeding cultures a yeast-Cerophyll™-trout chow (YCT) mixture together with the green algae *Selenastrum capricornutum* and the diatom *Navicula* spp., three times per week. The choice of food type and ration to be used for culturing *H. azteca* is left to the discretion and experience of laboratory personnel.

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[9] Continuous recirculation of culture water through commercial aquarium supply filters can also maintain good quality water within cultures (G.A. Burton Jr., Wright State Univ., Dayton, OH, personal communication, 1994), and such apparatus may be used as an alternative or supplement to water renewal.

2.3.9 Handling Organisms

Amphipods should be handled as little as possible. When handling is necessary, it should be done gently, carefully, and quickly to minimize stress to the animals. Adult or younger individuals can be transferred between containers using a glass or clear plastic pipette with a polished end and an opening of about 5 to 6 mm in diameter. Transferred organisms should be released below the water surface. When handled, any animals that are dropped, injured, contact dry surfaces, or appear stressed must not be used for testing.

2.3.10 Mixed Age and Known Age Cultures

There are various options for the type, size, and loading densities of culture chambers (see Appendix C.2), as well as the type of culture water used and its method of replacement (Appendix C.3), choice of culturing substrate (Appendix C.5), food type and feeding frequency during culturing (Appendix C.6), and techniques for harvesting young for tests (Appendix C.7). For this test method, such choices are left to the discretion and experience of laboratory personnel; however, the culturing procedures used must produce enough 2- to 9-day old amphipods to start the planned toxicity tests with sediment and/or reference toxicant(s). Additionally, the cultured organisms must meet specific performance criteria (see Sections 2.3.11, 4.2, and 4.8).

Laboratories culturing *H. azteca* frequently maintain both *mixed age* and *known age* cultures, and such practice is recommended here. The mixed age culture(s) could represent a mass culture of amphipods of various ages, maintained in one or more aquaria (see Appendix C.2). The *known age* cultures contain individuals of a particular age class (e.g., <1- to 7-day old or 7- to 14-day old) that have been segregated and maintained in a number of aquaria, jars, or other culture chambers (Appendix C.2) until they are used in toxicity tests. Various procedures exist for culturing known age individuals (USEPA, 1994a), and those that work for laboratory personnel may be used to provide test organisms. In each of these procedures, the water in the culture chambers is changed routinely and thus known age animals are obtained.

Following is a procedure (Hamr *et al.*, 1994; Milani *et al.*, 1996) for generating <1- to 7-day old *H. azteca*, and for holding them for a subsequent 2-day period (i.e., until they are 2- to 9-days old) before their use in a 14-day sediment toxicity test. Hamr *et al.* (1994) provide a rationale and experimental data that support this choice of age of test organisms and a 14-day test duration. The procedure for obtaining animals within this age range has been modified from Borgmann *et al.* (1989). This procedure is recommended as one of several ways of obtaining adequate numbers of 2- to 9-day old organisms (at the time they are used in the sediment toxicity tests).

Approximately 150 adult amphipods that are ≥30-d old and ideally in amplexus are placed in 20-L culture chambers, each containing about 15 L of culture water. Each chamber contains pieces of cotton gauze. The cotton gauze should be presoaked in water for 24 h before its initial use, and can be used for up to three weeks before replacement. A combined yeast-Cerophyll™-trout chow (YCT) preparation (see Appendix G) is provided daily to each culture chamber. Once a week, the test organisms are isolated from the gauze and the culture water by pouring the contents of the culture chamber(s) through 500 µm and 250 µm sieves. The animals retained by the 500 µm sieve are returned to the culture chambers, which are replenished with at least 30% fresh water and food. The animals retained by the 250 µm sieve are <1- to 7-days old. These animals are rinsed into a translucent white plastic pan placed on a light table, and are counted. The young amphipods from each 20-L culture chamber are then transferred, using a pipette, into a 1-L beaker, which contains 750 mL of fresh culture water. Density of amphipods in beakers should not exceed 1 animal per 10-mL volume of culture water (i.e., 75 animals/beaker) to avoid growth inhibition (K. Day, NWRI, Burlington, ON; unpublished data). Presoaked cotton gauze should be placed in the beaker to provide a substrate for the animals. Organisms in each 1-L beaker are fed 10 mL of YCT daily, and are held for 2 days before starting a toxicity test. Accordingly, the animals are 2- to 9-days old at the start of the test. Each culture chamber containing 150 animals will produce 100 to 150 young per week, on average.

A narrower age range (e.g., ± 1 day) of organisms used to start a test might be desirable, especially since growth is measured as a primary test endpoint. A technique is described by USEPA (1994a) where mature amphipods are separated by a #25 (710µm) sieve as previously described, and the sieve is held in a glass pan containing culture water overnight. Any newborn amphipods that are released after 24 h are collected by moving the sieve up and down several times to rinse the newborns into the surrounding water in the pan. The sieve is then removed from the pan and mature amphipods are returned to the mixed age culture. The newborn amphipods that passed through the sieve are transferred using a pipette, and are placed in a culture chamber for a grow-out period. The newborn amphipods should be counted to determine whether adequate numbers have been collected for the test. Isolation of about 1500 (750 pairs) adults in amplexus will provide about 800 newborn amphipods in 24 h, and requires about six person-hours of time.

Records should be kept on the number of surviving adults, number of breeding pairs, and the number of young produced and their survival. Records should also be kept on the age of brood organisms, and on the frequency of restarting cultures. This information can be used to develop performance charts which are useful in determining whether cultures are maintaining a vigorous reproductive rate indicative of culture health. Some of the adult amphipods can be expected to die in the culture chambers, but excess mortality should be cause for concern. A decrease in reproductive rate could be caused by a change in water or food quality, or by deteriorating health of the brood stock. Culture performance is affected by the age of adults, and can be cyclical. Adult females will continue to reproduce for several months; however, fertility gradually decreases after about three months (USEPA, 1994a).

2.3.11 Health Criteria

Amphipods in the cultures should be checked three times per week (e.g., Monday, Wednesday, Friday) as a minimum, and preferably daily. Individuals that appear dead or inactive when gently prodded must not be used for testing. If more than 20% of the amphipods in a *known age* culture chamber appear dead or inactive during the 48-h period preceding the start of the test, the entire group in the container must be discarded (USEPA, 1994a).

Ideally, a reference toxicity test should be performed in conjunction with each sediment toxicity test. Laboratories routinely undertaking sediment toxicity tests using *H. azteca* may choose to conduct reference toxicity tests once each month, according to a regular schedule. All tests with reference toxicants should be performed using the conditions and procedures outlined in Section 4.8. Test related criteria used to judge the health and sensitivity of the culture are given in Sections 4.2 and 4.8.

Biochemical measurements such as the lipid content of cultured amphipods, or the average number of young produced in a week by each adult in a culture, might provide useful information on the health of the cultures (USEPA, 1994a). Ongoing records of these or other indices of the condition of cultures will likely prove useful, and are encouraged. No specific health criteria have been developed as yet with respect to physiological measurements, although they could be applied in the future.

Test System

3.1 Facilities and Apparatus

Tests may be performed in a water bath, environmental chamber, or equivalent facility having acceptable temperature and lighting (see Section 3.2) control. The test facility must maintain the daily mean temperature of all sediment and water in test chambers at 23 ± 1°C (see Section 4.2). The facility should be well ventilated to prevent exposure of personnel to harmful fumes, and isolated from physical disturbances or any contaminants that might affect the test organisms. The area used to manipulate sediment in preparation for tests should also be properly ventilated.

The test facility should be isolated from the area where amphipods are cultured, to avoid potential contamination of cultures. Additionally, the test facility should be removed from places where samples are stored or prepared, to prevent the possibility of contamination of test chambers and their contents from these sources. The ventilation system should be designed, inspected, and operated to prevent air within the testing facility from contaminating culture facilities. Return air from sample handling and storage facilities or those where chemicals are processed or tested should not be circulated to the area of the laboratory where tests are conducted. Any construction materials that might contact the organisms, water, or test chambers within this facility must be nontoxic (see Section 2.3.2).

Compressed air used within the test facility for aerating water must be free of oil and fumes. Oil-free air pumps should be used wherever possible. Any oil or particulate in the air supply should be removed by online filters, which are replaced as required to ensure their effectiveness.

Equipment and supplies that contact water, sediment, or test chambers should be chosen to minimize sorption of chemicals. Borosilicate glass, nylon, high-density polyethylene, polycarbonate, fluorocarbon plastics, and type 316 stainless steel should be used whenever possible to minimize chemical sorption and leaching. The use of apparatus and supplies made of toxic substances (see Section 2.3.2) must be avoided.

The test method may be performed as either a daily renewal test, or as a static test (see Section 4). If the daily renewal test option is selected, apparatus used to deliver and renew overlying water in test chambers may be one of several designs[10] (e.g., Maki, 1977; Benoit *et al.*, 1993; Zumwalt *et al.*, 1994). A suitable apparatus would be one that enables the timed and intermittent renewal of the overlying water in each test chamber at a rate of 2 volume additions/day (USEPA, 1994a). The automated water-renewal system designed by Zumwalt *et al.* (1994) is recommended, since it is inexpensive, easy to build and calibrate, and delivers small volumes of water to test chambers with minimal variation. The USEPA (1994a) provides useful guidance on the design of this and other suitable systems for the automated renewal (at a rate of 2 volume additions/day) of overlying water.

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[10] The overlying water may also be replaced manually, by siphoning. Manual systems, however, are more labour intensive and generally result in more suspension of sediment compared to automated systems (USEPA, 1994a).

If the static test option is chosen, or for daily-renewal tests requiring aeration of the overlying water (see Section 4.3), a supply of disposable glass pipets and aquarium supply airline tubing is required for delivery of a continuous (gentle) flow of air to each test chamber. Stainless-steel (rather than brass) gang valves are recommended for regulating air flow. For static tests (including those using reference toxicant), a supply of suitably sized watchglasses or lids is needed for covering individual test chambers.

The test facility must have the basic instruments required to monitor the quality (e.g., temperature, pH, dissolved oxygen, conductivity) of the test water and pore water. Additionally, the laboratory should be equipped to facilitate prompt and accurate analysis, with acceptable limits of detection, of such variables as hardness, alkalinity, ammonia, and (in instances where dechlorinated municipal water is used as culture or test water) residual chlorine.

All test chambers, equipment, and supplies that might contact sediment or test water, must be clean and rinsed with test water, deionized water, or distilled water, before its use. All nondisposable materials should be washed after use. The following cleaning procedure (USEPA, 1994a) is recommended.

1. Soak in tap water for 15 minutes, then scrub with detergent or clean in an automatic dishwater.

2. Rinse twice with tap water.

3. Rinse carefully with fresh, dilute (10%, v:v[11]) nitric (HNO_3) or hydrochloric acid (HCl) to remove scale, metals, and bases.

4. Rinse twice with deionized water.

5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).

6. Rinse three times with high-quality deionized water.

Test chambers and apparatus that might contact sediment or test water should be thoroughly rinsed with test water, immediately before use in the test.

Before toxicity tests are undertaken in a new test facility, a minimum of five, 96-h *water only* reference toxicity tests, and a minimum of five, 14-day *survival-and-growth* tests with control sediment, should be undertaken to confirm that acceptable performance of *Hyalella azteca* can be achieved using the new facility and the culturing and test conditions and procedures specified in this report (see Sections 2.3.1 and 4). Each test with reference toxicant or control sediment should be performed using a different batch of cultured organisms. Data from these preliminary tests should be compared by calculating and appraising the magnitude of the coefficient of variation for the respective series of tests and endpoint values.

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[11] To prepare a 10% solution of acid, carefully add 10 mL of concentrated acid to 90 mL of deionized water.

3.2 Lighting

All test chambers should receive full-spectrum (e.g., fluorescent or equivalent) illumination from directly overhead, at an intensity sufficient to provide 500 to 1000 lux adjacent to the surface of overlying water in test chambers. Illumination should be as uniform as possible for all test chambers. Photoperiod should be regulated at 16-h light and 8-h dark.

3.3 Test Chambers

High form glass beakers or glass jars with a capacity of 300 mL and an inner diameter of ~7 cm are recommended as test chambers (USEPA, 1994a; ASTM, 1995a; EC, 1997a). Each beaker or jar must be cleaned thoroughly before and after use (Section 3.1), and rinsed well with test water immediately before use. Covers for test chambers are not normally required for daily renewal tests, although they should be used for static tests and are also recommended for daily renewal tests if test sediments contain detectable volatile gases. Suitable covers include clean watch glasses, or glass or plastic lids.

3.4 Test and Control/Dilution Water

Depending on the test design and intent (see Sections 5 and 6), test water (i.e., water overlying sediment in the test) and control/dilution water (i.e., water used to prepare dilutions of test chemicals and as control water in *water only* exposures with reference toxicants) may be either an uncontaminated supply of natural fresh or estuarine water, or reconstituted water. The water supply used as test or control/dilution water is frequently the same as that used for culturing *H. azteca* (see Section 2.3.4), although it may come from another source. For instance, the use of site water, or clean water adjusted to the hardness of water at a collection site, might prove a good choice (see Section 5.4). The quality of test water and that used as control/dilution water is extremely important; this water must have been demonstrated to allow acceptable survival and growth of test organisms in 14-day tests with control sediment (see Section 4.2) before it is used in toxicity tests. Guidance for preparing reconstituted fresh water or estuarine water (salinity ≤15 ‰) is provided in Section 2.3.4.

Test and control/dilution water must be adjusted to the test temperature (23 ± 1°C) before use. The dissolved oxygen content of the water should be 90 to 100% of the air saturation value at this temperature. As necessary, the required volume of water should be aerated vigorously (oil-free compressed air passed through air stones) immediately before use, and its dissolved oxygen content checked to confirm that 90 to 100% saturation has been achieved. The pH of the water should be measured and stable before use.

3.5 Control Sediment

Each sediment toxicity test must include an experimental control, with a minimum of five replicate beakers or jars containing control sediment. A control sediment is a sediment that is essentially free of any contaminants that could adversely affect the survival, growth, or behaviour of *H. azteca* during the test. The use of control sediment provides a measure of test acceptability (i.e., mean survival at test end must be ≥80%), evidence of the health and behaviour of the test organisms, and a basis for interpreting data derived from the test sediments.

Control sediment may be either natural sediment taken from a collection site removed from known sources of contaminants and shown previously to enable acceptable survival and growth of *H. azteca* under the conditions of the test, or formulated sediment.[12] The selection of an appropriate control sediment depends on considerations such as the study design, physicochemical characteristics of the test sediment(s), and the availability of suitable *clean* sediment having the desired properties. There should also be evidence that the sediment provides consistent and acceptable biological endpoints using *H. azteca* and this test method. While many clean, natural sediments have been used as control sediment in toxicity tests with *H. azteca*, the use of formulated control sediment offers a more consistent, standardized approach and one which numerous researchers are now actively pursuing (Smith *et al.*, 1992b; Dwyer *et al.*, 1993; Suedel and Rodgers, 1994a; b; USEPA, 1994a; Suedel *et al.*, 1996). At present, it is premature to recommend a recipe for formulated control sediment that is suitable for a 14-day *survival-and-growth* test using *H. azteca*.

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[12] Formulated sediment is also described as reconstituted, artificial, or synthetic sediment. It is typically prepared using sand, silt, clay, and nontoxic organic constituents obtained from commercial sources, and is hydrated with reconstituted or natural water. Formulated sediment can be prepared to match different natural sediments with respect to particle size distribution, organic carbon content, pH, cation exchange capacity, etc. (Suedel and Rodgers, 1994b; Milani *et al.*, 1996). Alternatively, one or more recipes can be used to prepare standardized control sediment(s) for routine use in freshwater sediment toxicity tests with *H. azteca* or other infaunal species (Suedel *et al.*, 1996).

Universal Test Procedures

General procedures and conditions herein apply to each of the described toxicity tests for samples of sediment, particulate waste, or chemical, and to reference toxicity tests. More specific procedures for conducting tests with field-collected samples of sediment or other similar particulate material (e.g., sludge, dewatered mine tailings, drilling mud residue) are provided in Section 5. Guidance and specific procedures for conducting tests with control or other sediment spiked experimentally with chemical(s), contaminated sediment, or particulate waste are given in Section 6.

All aspects of the test system described in Section 3 must be incorporated into these universal test procedures. The summary checklist of recommended test conditions and procedures in Table 2 describes not only universal procedures but also those for testing specific types of test substances or materials.

Universal procedures are described herein for performing a 14-day[13] sediment toxicity test, which includes the following two test options[14]:

(1) a daily-renewal test, where the overlying water is renewed at a rate of two volume additions/day, and is normally not aerated; and

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[13] Toxicity tests with *H. azteca* of up to four weeks' duration have commonly been performed (e.g., Nebeker *et al.*, 1984; Borgmann and Munawar, 1989; Borgmann and Norwood, 1993; Ingersoll and Nelson, 1990; ASTM, 1991a; 1993; NWRI, 1992). Extending the test duration beyond 14 days could enhance the test's ability to discern toxic effects on survival of amphipods; however, the sensitivity of the growth endpoint might not improve due to sexual maturation and associated dimorphism in size of males and females which occurs at this time (Borgmann *et al.*, 1989). Using the conditions and procedures described here, or, as required, appropriate modifications thereof, the test could also be continued beyond four weeks to discern and measure effects on reproduction of *H. azteca* (Borgmann *et al.*, 1989; ASTM, 1991a; 1993).

[14] Similar results are apparently obtained by static and static-renewal tests, when performed according to the procedures defined herein. The performance of these two test options was compared in side-by-side tests using samples of field-collected or contaminant-spiked sediment (Milani *et al.*, 1996). Results for interlaboratory tests with *H. azteca* indicated that test precision and sensitivity were similar using either system (Milani *et al.*, 1996). Interlaboratory coefficients of variation (CVs) for grand means (all laboratories) of the 14-day survival data for each of four samples of field-collected sediment ranged from 3.6 to 19.6% using the static system, and from 2.5 to 11.0% using the static-renewal system. Data for growth were more variable in both systems, with CVs for dry weight of amphipods at test end ranging from 28.4 to 48.8% using the static system and from 26.0 to 35.7% using the static-renewal system.

Results for tests conducted by each laboratory using a range of concentrations of copper-spiked sediment showed a trend toward lower LC50s (survival data) and lower IC25s (growth data) using the static option, although these differences (indicating greater test sensitivity using the static system) were significant for only two of the four laboratories conducting side-by-side tests with each option (Milani *et al.*, 1996).

Table 2 **Checklist of Recommended Conditions and Procedures for Conducting Sediment Toxicity Tests Using *Hyalella azteca***

Universal

Test type	–	14-day whole sediment toxicity test with two options: daily renewal of overlying water; or no renewal (static test)
Water renewal	–	if using daily-renewal option, overlying water is replaced at a rate of two volume additions/day; if using static option, no renewal of overlying water during test except for replacement for losses due to evaporation
Test (overlying) water	–	culture water or other clean ground or surface water; site water; water adjusted to hardness of site water; reconstituted fresh water for higher degree of standardization; natural or reconstituted seawater with salinity ≤ 15‰ for tests with estuarine sediment; dissolved oxygen, 90 to 100% saturation when used as overlying water in test
Control sediment	–	sample of clean sediment that is used to assess the performance of the test organisms and the acceptability of the test; either natural or formulated sediment
Amphipods	–	removed from known age culture as <1- to 7-d old individuals and held in 750 mL of culture water within 1-L beaker for 2 d preceding test, while fed 10 mL YCT daily; test organisms 2- to 9-d old at start of test; 10 animals/test chamber
Test chamber	–	300-mL high form glass beaker or glass jar; ~7 cm inner diameter; normally uncovered if daily-renewal test and covered if static test
Volume of wet sediment	–	100 mL
Volume of test water	–	175 mL
Number of replicates	–	recommend ≥5 field replicates, each a discrete (i.e., different) sample from the same location; must be ≥5 laboratory replicates for each field replicate
Temperature	–	daily average, 23 ± 1°C; instantaneous, 23 ± 3°C

Lighting	-	overhead full-spectrum (fluorescent or equivalent); 500 to 1000 lux; 16-h light:8-h dark
Aeration	-	if using daily-renewal option, none unless dissolved oxygen in overlying water drops below 40% of saturation; if using static option, continuous and minimal (e.g., 2 to 3 bubbles/s, each test chamber)
Feeding	-	aqueous suspension of YCT, fed daily or three times per week (nonconsecutive days); 1.5 mL (~2.7 mg solids, dry weight) added daily to each test chamber if daily feeding; 3.5 mL (~6.3 mg dry solids) added each feeding to each test chamber if fed three times per week only
Observations	-	optional: numbers of amphipods in each chamber seen emerged from sediment, and their behaviour (daily or less frequently)
Measurements of overlying water	-	≥3 times/week, each treatment, for DO and temperature; start and end of test, each treatment, for pH, conductivity, and ammonia; recommend hardness and/or alkalinity at start and end
Endpoints	-	significantly lower survival and weight than in control or reference treatments (based on mean percent survival and mean dry weight, each treatment); 14-d LC50 for multi-concentration test, where appropriate; ICp for weight, where appropriate
Test validity	-	invalid if mean 14-day survival in control sediment <80%; invalid if average dry weight for replicate control groups at test end is <0.1 mg/amphipod

Field-collected Sediment or Similar Particulate Material

Transport and storage	-	if sample >7°C, cool to 7°C (ice or frozen gel packs); transport in dark at 1 to 7°C (preferably 4 ± 2°C); store in dark at 4 ± 2°C; test should start within two weeks and must start within six weeks

Reference sediment –	one or more samples for tests with field-collected sediment; taken from sites presumed to be clean but in the general vicinity of sites where test sediments are collected (i.e., same body of water); frequently selected for use in the toxicity test because of its physicochemical similarity (e.g., particle size and/or organic carbon content) to the test sediments
Sample characterization –	at least particle size analysis (percent sand, silt, and clay), total organic carbon, percent water, porewater pH, and porewater ammonia
Preparation of sample –	only if necessary, remove debris and indigenous macro-organisms using forceps; homogenize sample (including any separated liquid) before the test; if necessary, remove smaller macro-organisms by pressing through fine-mesh sieve (e.g., 0.25 to 0.5 mm), or pass through fine-mesh sieve using liquid that separated from sample during transit and storage and remix this liquid with the sieved sample

Spiked Sediment

Characterization of chemical(s) added –	information required on stability, water solubility, vapour pressure, purity, and biodegradability should be known for chemicals spiked into control sediment
Solvent –	test water is the preferred solvent; if an organic solvent is used, the test must include a solvent control
Preparation of mixtures –	procedure depends on test design and objectives; might include one or more chemical concentrations mixed in control or test sediment, or specific chemical concentrations added to the test water overlying control sediment; chemical/sediment mixtures may be prepared manually or by mechanical agitation as slurries
Concentration of chemical(s) added –	normally measure at beginning and end of test, in high, medium, and low strengths as a minimum
Test and dilution water –	use reconstituted water if a high degree of standardization is required

(2) a static test, where the overlying water is not renewed during the test, but is aerated.

Using either test option, the amphipods are fed an aqueous suspension of YCT, either as a daily inoculum of 1.5 mL (~2.7 mg food, dry weight) or three times per week on nonconsecutive days using an inoculum of 3.5 mL (~6.3 mg dry weight). Biological endpoints measured in this test method are survival and dry weight at test end.

4.1 Beginning the Test

Each test chamber (see Section 3.3) placed within the test facility must be clearly coded or labelled to enable identification of the sample or its concentration. The date and time when the test is started must be recorded, either directly on the labels or on separate data sheets dedicated to the test. The test chambers should be positioned for ease while making observations and measurements. Treatments should be positioned randomly within the test facility (USEPA, 1994a).

The day that amphipods are initially exposed to samples of test materials or substances is designated Day 0. On the day preceding the start of the test (i.e., Day -1), each sample or subsample of test sediment or similar particulate material, including control and reference sediment, should be mixed thoroughly[15] (see Sections 5.3 and 6.2) to provide a homogeneous mixture consistent in colour, texture, and water content. Quantitative measures of homogeneity might include particle size analysis, total organic carbon, percent moisture, and concentration of specific chemicals.

Immediately following mixing, replicate 100-mL volumes of the sample should be transferred to the test chambers. A minimum of five laboratory replicates per treatment must be established[16] (see Sections 5.1 and 6.2). The sediment added to each chamber should be smoothed using a spatula or by tapping the chamber against the side of the hand. Test water (see Section 3.4) is then poured slowly down the side of the beaker or jar. To minimize the disruption of sediment as test water is added, a disc made of Teflon™, polyethylene, or nylon sheeting, cut to fit the inside diameter of the test chamber, may be placed on the sediment surface before water is added[17] (EC, 1992a). For tests with daily renewal of the water overlying sediment, a total volume of 175 mL of test water (or, depending on the test, a test solution) should be added to the test chamber at this time; this can be judged using a 275-mL mark inscribed on the chamber's side. For static tests, a somewhat lesser volume (e.g., 125 to 145 mL) of test water may be added initially, to provide room for any additional water added when test organisms are introduced.

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[15] Any liquid that has separated from the sample during transport and/or storage must be remixed within the sample.

[16] USEPA (1994a) indicates a minimum requirement of four replicates per treatment, and recommends eight replicates per treatment for sediment-toxicity tests.

[17] A length of nylon monofilament line (or nontoxic equivalent) could be attached to the disc, to enable its removal once the test water is added. Alternatively, the disc could be cut from a polyethylene bag in a keyhole configuration which provides a circle with an attached portion for removal. The disc should be rinsed with test water if reused to prepare replicates of a treatment. A separate disc should be used for each treatment.

If the daily-renewal test option is chosen, water overlying the sediment in each test chamber should be renewed on the day preceding the test (Day-1) as well as throughout the test, at a rate of two volume additions per day[18] (USEPA, 1994a; Table 2). A replacement rate greater than two volume additions per day should be avoided to prevent unnecessary flushing and depletion of any contaminants that might leach from the sediment into the overlying water. The overlying water is normally not aerated and test chambers are not covered, using this test option. The use of an automated intermittent-renewal system such as the one designed by Benoit *et al.* (1993) and modified by Zumwalt *et al.* (1994), is recommended (see Section 3.1); this apparatus can be set up to renew the overlying water at 12-h intervals. If an automated system is used, it should be calibrated before the test is started to verify its performance; flow rates through any two test chambers should not differ by more than 10% at any time during the test (USEPA, 1994a; ASTM, 1995a). If overlying water is renewed twice daily by siphoning, care should be taken to prevent disturbance of the sediment or accidental loss of amphipods emerged from the sediment during this procedure. No more than 90% of the water should be siphoned and replaced, and the end of the siphon must not contact the sediment.

If the static test option is chosen, the overlying water in each test chamber should be aerated overnight before the test organisms are introduced, as well as throughout the test (see Section 4.3). Each beaker or jar should be kept covered (watchglass or plastic lid) during the pretest and test periods, to minimize evaporation and to reduce the possibility of contamination. Any overlying water lost by evaporation should be replaced on Day 7 of the test (or more frequently, if desired or necessary) by the gentle addition of temperature-adjusted test water poured down the side of the test chamber. A 275-mL mark inscribed on the side of the test chamber can be used to judge this.

Test organisms used to begin the test are those that are 2- to 9-days old on the day that the test is initiated (see Section 2.3). In many instances, the culture water and the water used as overlying water in the test will be the same, although this is not necessarily so. The objectives of a particular test might require the use of another water source (e.g., that from a particular site under investigation) as test water. If water other than culture water is used as the test water, acclimation of test organisms to this water is not required (USEPA, 1994a) although it might be advisable to do so in order to minimize any stress on the animals caused by different water quality characteristics. If test organisms are to be acclimated, a useful procedure is to hold them for 2 h in a 50:50 mixture of culture water:test water, then for 2 h in a 25:75 mixture of culture water:test water, followed by a final 2 h in 100% test water before their introduction to test chambers (Ingersoll and Nelson, 1990). This should be done on the day before the test starts.

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[18] In water-renewal tests with one to four volume additions of overlying water/day, water quality characteristics generally remain similar to the inflowing water. However, in sediment toxicity tests where overlying water is not renewed or is replaced less frequently than once per day, the quality of the overlying water (e.g., conductivity, alkalinity, hardness) can change markedly. For instance, concentrations of hardness, alkalinity, conductivity, or metabolic products (e.g., ammonia) in the overlying water might increase (Ingersoll and Nelson, 1990; Ankley *et al.*, 1993a; USEPA, 1994a). Such changes in water quality characteristics could influence the test results, and can be avoided by the routine (i.e., twice daily) replacement of overlying water throughout the test period. Regardless, certain researchers prefer the static test option, due to the lesser labour and equipment requirements and the possibility that a daily- (or twice-daily) renewal test might deplete toxicants from the sediment during the test and perhaps reduce its potential toxic effects (Milani *et al.*, 1996).

If toxicity tests are intended using samples of estuarine sediment, it is recommended that the test organisms be acclimated gradually to estuarine water with a salinity similar to that of the pore water of the test sediments, before the start of the test. Alternatively, additional controls could be included in the study, using control sediment with a porewater salinity similar to that of the test sediments.

On Day 0, ten amphipods should be assigned randomly to each test chamber. These organisms should be handled as little and as carefully as possible (see Section 2.3.9) during their transfer (Section 2.3.10) to the test chambers. Amphipods must be placed below the air/water interface in the overlying water. Test organisms may be pipetted directly from a culture chamber into the overlying water (Ankley *et al.*, 1993a). Alternatively, 10 amphipods may be counted into a transfer chamber (e.g., 30-mL plastic cup) filled with test water at the test temperature, and then recounted before their transfer below the surface of the overlying water (Ingersoll and Nelson, 1990; USEPA, 1994a). The latter procedure is particularly useful, since it permits the organisms to be counted twice before they are introduced to the test chamber. Following the addition of test organisms, the volume of water overlying the sediment should be increased as necessary until the 275-mL mark inscribed on the chamber's side is reached.

4.2 *Test Conditions*

- This is a whole sediment toxicity test, during which the overlying water is:
  - (1) not renewed except for the periodic (e.g., on Day 7) addition of required volumes of test water to replace that lost from evaporation (i.e., static test option); or
  - (2) is renewed throughout the test at a rate of two volume additions per day (i.e., daily-renewal test option).
- Test duration is normally 14 days.[19]
- The test must be conducted at a daily mean temperature (overlying water) of 23 ± 1°C. Additionally, the instantaneous temperature must be 23 ± 3°C (USEPA, 1994a; ASTM, 1995a).
- The recommended test container is a 300-mL high-form glass beaker or glass jar, with an inner diameter of
- ~7 cm.
- Control and test sediments must be present as a uniform, 100-mL layer, with a 175-mL volume of overlying water.
- For the daily-renewal test option, test chambers are normally uncovered,

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[19] See footnote 13.

although covers may be used to minimize loss of volatiles from the sediment or to reduce the risk of contamination. Overlying water is normally not aerated unless its concentration of dissolved oxygen drops below 40% saturation at any time during the test (see Section 4.3). Using this test option, amphipods in each test chamber must be fed either three times/week (nonconsecutive days) throughout the test, or daily (see Section 4.4).

- For the static test option, test chambers should be covered. The overlying water in each chamber should be aerated continuously at a minimal rate (see Section 4.3). Organisms in each test chamber must be fed either three times per week or daily (see Section 4.4).

- Test chambers are to be illuminated with a daily photoperiod of 16-h light and 8-h dark, using overhead, full-spectrum lights (fluorescent or equivalent). Light intensity adjacent to the surface of the overlying water should be 500 to 1000 lux.

- For a valid test, the mean survival rate for amphipods in control sediment must be ≥80% at the end of the test. Additionally, the minimum dry weight for the replicate control groups at test end (Day 14) must average ≥0.1 mg per individual amphipod.[20]

4.3 *Dissolved Oxygen and Aeration*

H. azteca can tolerate hypoxic conditions (Section 1.4). Using the daily-renewal test option, aeration of the overlying water is normally not required. The dissolved oxygen concentration in the overlying water will not be below 40% saturation due to its daily renewal, unless the sediment sample used in the test has an unusually high oxygen demand. If at any time during a daily-renewal test the dissolved oxygen is below 40% saturation or above 100% saturation in one or more test chambers, the overlying water in all test chambers including the controls should be aerated to maintain its dissolved oxygen concentration between 40% and 100% saturation. If the overlying water is to be aerated, oil-free compressed air should be dispensed to each test chamber through airline tubing and a disposable plastic or glass tube (e.g., capillary tubing or a pipette having an Eppendorf tip) with a small aperture (e.g., 0.5-mm ID). Stainless-steel gang valves are useful for regulating air flow. The tip of the air delivery tube should be suspended approximately 3 cm above the surface of the sediment layer. Air flow to each test chamber must be gentle (e.g., 2 to 3 bubbles/s), and must not disturb the sediment surface (Zumwalt *et al.*, 1994). Any aeration during testing must be reported (Section 7).

Using the static test option, the overlying water in each test chamber should be aerated continuously on the night preceding the start of the test (see Section 4.1), as well as during the test. Compressed air, previously filtered so as to be free of oil, should be bubbled through a disposable glass pipette. The tip of the pipette should be suspended approximately 3 cm above the surface of the sediment layer. Air flow to each test chamber must be gentle and regulated to provide approximately 2 to 3 bubbles/s. The air flow to each test chamber should be checked routinely (e.g., daily) throughout the test, and adjustments made if necessary to maintain a gentle rate of aeration.

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[20] Based on a review of dry weight attained by control groups in 14-day tests with *H. azteca*, under static or static-renewal conditions defined in this report, Milani *et al.* (1996) concluded that a criterion for test validity of ≥0.1 mg per individual control organism would normally be attainable yet discriminatory for this species, and recommended this for inclusion as a test criterion using either option.

4.4 Food and Feeding

Using either test option, organisms in each test chamber must be fed either once daily throughout the test, or three times weekly (on nonconsecutive days) throughout the test. Since dry weight of amphipods is a primary endpoint for the test, an identical food ration must be added to each test chamber on each feeding occasion. The ration provided must be adequate to enable acceptable survival and growth of *H. azteca* during the test period (see Section 4.2), but must not be excessive.[21]

If daily feeding is chosen, an inoculum of 1.5 mL (equivalent to ~2.7 mg food, dry weight) of a mixture of yeast, Cerophyll™, and trout chow[22] (YCT) must be added daily to each test chamber on Day 0, as well as once per day thereafter until the day the test ends. If the option of feeding three times per week is chosen, an inoculum of 3.5 mL YCT (equivalent to ~6.3 mg food, dry weight) must be added three times per week (starting on Day 0) to each test chamber on nonconsecutive days (e.g., on Mondays, Wednesdays, and Fridays) until the day the test ends. Either ration results in the same overall rate of feeding; i.e., 10.5 mL YCT (~18.9 mg dry food) weekly, per test chamber. Daily feeding is preferable to "even out" the available food supply, although feeding three times per week might be a preferred choice to minimize weekend labour requirements (Milani *et al.*, 1996).[23]

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[21] Feeding during the test is essential to enable adequate (≥80%) survival and acceptable growth of test organisms (Ankley *et al.*, 1993a; 1994; Milani *et al.*, 1996). The addition of excess or different types of food is to be avoided since it might alter the bioavailability of contaminants in the sediment and/or promote the growth of fungi or bacteria on the sediment surface (USEPA, 1994a).

[22] This food type and ration has proven suitable for *H. azteca* under the defined test conditions (Ankley *et al.*, 1993a; 1994; Milani *et al.*, 1996), and the daily ration represents that recommended in USEPA (1994a) and ASTM (1995a) for sediment toxicity tests using *H. azteca*. Other food types and rations, including single ration diets of rabbit chow (Ingersoll and Nelson, 1990; ASTM, 1991a; 1993), commercial fish food flakes (Borgmann *et al.*, 1989; NWRI, 1992), or multiple ration diets such as algae plus alfalfa plus fish food flakes (Herrin *et al.*, 1992), have been shown previously to enable adequate (≥80%) survival and acceptable growth of control animals using the conditions and procedures specified for this test. However, the use of a food type or ration other than that specified here (i.e., YCT, fed daily as 1.5mL/test chamber/feeding or three times per week as 3.5 mL/test/chamber/feeding) is not recommended, since such differences could alter the bioavailability of contaminants and reduce the standardization of the assay. See Appendix H for preparing YCT.

[23] Results for 14-day side-by-side comparisons using either feeding regime showed that survival and growth (dry weight at test end) of *H. azteca* did not differ significantly, regardless of whether the static or static-renewal options were used (Milani *et al.*, 1996).

Detailed records of the food type and ration added to each test chamber should be made on each feeding occasion. Observations of the appearance of the sediment surface in each test chamber (i.e., any evidence of a fungal or bacterial growth) should also be recorded at this time. If, using the daily-renewal test option, the dissolved oxygen concentration in one or more chambers drops below 40% saturation, feeding in all treatments should be temporarily suspended for the amount of time necessary for its recovery (USEPA, 1994a).

4.5 Observations and Measurements During the Test

If the daily-renewal test option is chosen and an automated water-renewal system is used (see Sections 3.1 and 4.1), its operation should be monitored daily. Any observations of water flow problems, or overflows in test chambers due to clogged drain screens, should result in immediate cleaning or other required maintenance.

Depending on the objectives, it might be worthwhile to regularly check each test chamber (preferably, daily), to observe and record the number of amphipods seen swimming in the overlying water, floating on the water surface, or lying or grazing on the surface of the sediment.[24] Any animals seen floating on the water surface should be gently pushed down into the water using a glass rod or pipette.

The temperature of the overlying water must be measured at the beginning of the test, and thereafter at least three times per week on nonconsecutive days (e.g., Mondays, Wednesdays, Fridays) until test completion. These measurements must be made in at least one test chamber representing each treatment; and more frequent (i.e., daily) measurements are recommended. Additionally, it is recommended that the temperature of any water bath used, and/or of the air in a temperature-controlled room or chamber used for the test, be recorded continuously.

For at least one test chamber representing each treatment, the concentration of dissolved oxygen in the overlying water must be measured at the beginning of the test, and thereafter at least three times per week on nonconsecutive days (e.g., Mondays, Wednesdays, Fridays) until test completion. More frequent (e.g., daily; ASTM, 1995a) measurements might be advisable and would be warranted for sediments having a high oxygen demand. A probe and calibrated dissolved oxygen (DO) meter is recommended for these measurements. The probe must be inspected carefully after each reading to ensure that organisms have not adhered to it, and must be rinsed in deionized or distilled water between samples to minimize cross-contamination. If beakers or jars are aerated during the test (Section 4.3), the position of the tip of the pipette in each test chamber and the rate of aeration should be checked frequently and routinely, and adjustments made as necessary.

Conductivity, pH, and ammonia concentrations in the overlying water must be measured at the beginning and end of the test for at least one test chamber representing each treatment. Additionally, hardness and/or alkalinity concentrations in the overlying water should be measured at the beginning and end of the test in at least one test chamber representing each treatment (USEPA, 1994a). For each measurement of ammonia (see APHA *et al.*, 1995 for guidance), the concentration of un-ionized ammonia should be calculated based on the concurrent measurements of pH and temperature for the overlying water (Trussell, 1972; USEPA, 1985c).

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[24] Records of numbers of animals emerged from the sediment might prove useful in assessing avoidance responses. However, since *H. azteca* is an epibenthic amphipod which frequently emerges from clean sediment, such observations are not necessarily worthwhile and are not required as part of this test method.

Conductivity and pH may be measured using probes and calibrated meters. Ammonia may be measured using an ion-specific electrode or by extracting an aliquot of the overlying water for this analysis. As with DO measurements, any probe inserted in a test chamber must be inspected carefully immediately after each reading, and rinsed in deionized water between samples. For measurements of hardness, alkalinity, and ammonia requiring sample aliquots, samples of overlying water should be taken just before the addition of test organisms, and upon completion of the test. For these samples, it might be necessary to pool water samples from individual replicates. No more than 10% of the volume of the overlying water in a test chamber (i.e., ≤17.5 mL) should be removed for this purpose. A pipette should be used carefully to remove water from a depth of about 1 to 2 cm above the sediment surface. The pipette should be checked to make certain that no amphipods are removed during the collection of these water samples.

The water quality measurements determined at the beginning and end of a test for each treatment are useful as they provide an indication of the influence of the sediment on overlying water quality during the test. If, for any treatment, a marked change (e.g., >50%; USEPA, 1994a) in one or more of these water quality variables is found between the initial and final measurements, a check on the conditions and procedures used in the test (e.g., rate of renewal of overlying water, if a daily-renewal test) is recommended, together with a careful consideration of the physicochemical characteristics of the sediment used in the test.

4.6 Ending a Test

The test is terminated after 14 days. Just before sieving the contents of a test chamber, all live and apparently dead amphipods in the water column or on the surface of the sediment should be pipetted from the test chamber. Individuals which are completely inactive but not obviously dead (e.g., not decomposing) should be held in test water within a petri dish or other suitable container, and examined closely at this time using a low-power microscope or hand-held magnifying glass. These individuals should be prodded gently with a sharp point to confirm that they show no sign of life (such as a pleopod twitch), and are then to be counted as dead.

Numbers of dead and surviving amphipods recovered by pipetting should be recorded and dead animals discarded. All live animals should be placed in a numbered weighing boat or similarly small holding receptacle containing sufficient test water for rinsing and holding the amphipods briefly until all of the survivors sieved from the sediment in the test chamber are added to and rinsed in it.

A consistent amount of time should be taken to sieve the contents of each test chamber and examine this closely for recovery of live or dead organisms. To ensure that the procedure used to recover amphipods is adequate, it is recommended that the laboratory personnel responsible for sieving the contents of test chambers previously demonstrate that they are able to retrieve an average of at least 90% of similar-sized *H. azteca* from sediment.[25]

The following technique, taken from USEPA (1994a), is recommended for sieving the contents of each test chamber. Other techniques or mesh sizes may also be used provided that they have been demonstrated in preliminary trials to allow the retrieval of test organisms.[26]

(1) Pour approximately 50% of the overlying water through a #50 (300 μm) U.S. Standard mesh sieve.

(2) Swirl the remaining water to suspend the upper ~1 cm of sediment. Pour the suspended slurry through the #50 mesh sieve. Using test water, wash the contents recovered on the sieve into a white tray or pan for inspection.

(3) Using test water and a wash bottle, rinse the coarser sediment remaining in the test chamber through a #40 (425 μm) mesh sieve and wash the contents recovered on this sieve into a second tray or pan for inspection.

All live animals recovered from the overlying water or sediment in a single test chamber are counted and placed together in a numbered weighing boat or similarly small holding receptacle, and rinsed in test water to remove any sediment adhering to the carapace of the animal. The rinse should be brief, and no more than 10 minutes following introduction of the first amphipod. After rinsing, the group of surviving amphipods should be transferred to a clean, aluminum weighing boat that has been previously numbered, weighed, and held in a desiccator.[27]

Separate weighing boats, each containing the group of surviving amphipods recovered from each test chamber (replicate), are placed in an oven, and dried for 24 h at 60°C (NWRI, 1992). Upon removal from the oven, the boats are moved immediately to a desiccator. Following cooling, each boat should be individually and randomly removed from the desiccator, and weighed immediately[28] to the nearest 10 µg on a balance that measures accurately to this limit. Mean dry weight per amphipod which survived the test is calculated for each group[29] (see Section 4.7).

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[25] USEPA (1994a) recommends a check on recovery capability used by Tomasovic *et al.* (1995), whereby test organisms are added to control sediment and their recovery determined after 1 h using the same technique as that employed for sieving the contents of test chambers at the end of the test.

[26] As an alternative to sieving, the sediment can be placed in one corner of a shallow, translucent tray on a light table. The tray is tilted, and the sediment washed downhill with a wash bottle and test water, to expose and count the amphipods (U. Borgmann, Fisheries and Oceans Canada, National Water Research Institute, Burlington, ON, personal communication, 1994).

[27] It might be advisable to oven-dry the weigh boats for at least 48 h to achieve a constant weight, since wax deposits associated with the weigh boats could otherwise provide weighing errors (G. Ankley, USEPA, Duluth, MN, personal communication, 1994).

During the series of dry-weight determinations for the groups of amphipods from a test, the first boat weighed should be replaced in the desiccator and weighed again at the end of all weighings, as a check on gain of water by the boats in the desiccator to be weighed subsequently. The change should not be >5%; if it is, redrying of all boats for ≥2 h and reweighing might be carried out. A few weighing boats should be tared, dried, and weighed without amphipods, and results should conform to the laboratory's quality control standards.

4.7 Test Endpoints and Calculations

The biological endpoints for this 14-day sediment toxicity test are survival and dry weight. Reduced survival and/or lesser weight at test end is assessed by comparison with replicate reference and/or control groups (see Sections 5.6 and 6.5). The most sensitive of the two effects is taken as the definitive indication of toxicity.

At the end of the 14-day exposure, the number of amphipods alive and number dead are recorded for each replicate including the control groups. The following two endpoints must be calculated for each treatment:

- the mean (± SD) percentage of amphipods that survived during the exposure.
- the mean (± SD) dry weight per surviving amphipod, calculated from the total weight of the group of survivors.

Missing individuals are assumed to have died and disintegrated during the test, and are included in the tally of dead individuals for a replicate. The total dry weight of the group of survivors in a replicate is then measured.

The test is invalid if the average percent survival for amphipods held in the control sediment for 14 days is <80% at the end of the test. The test is also invalid if the average dry weight for the replicate control groups is <0.1 mg per individual amphipod surviving at the end of the test.

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[28] The dried amphipods can take up water vapour readily, so weighing should be rapid and the time standardized among boats. At the same time, care must be taken because rapid movement and static charge could cause dried specimens to be lost from the weighing boat.

[29] The body length of individual amphipods surviving at the end of the test has been used as an alternative measurement of growth in sediment toxicity tests with *H. azteca* (see Appendix D.9). USEPA (1994a) endorses determinations of either dry weight or body length as endpoints representing growth. Herein, dry weight is the recommended indicator of growth. Measurement of body length offers some additional advantage over dry-weight measurements, in that specimens can be preserved for subsequent analyses (USEPA, 1994a) and data derived from individuals can be used for nested ANOVA and for appraising sexual maturation (Kemble *et al.*, 1994). Measurements of length can be substituted in this test for dry-weight measurements provided that future studies demonstrate conclusively that length is as, or more sensitive, an indicator of growth. Results by Becker *et al.* (1995) provide supporting evidence in this regard. In Environment Canada's *survival-and-growth* test using fathead minnows (EC, 1992c), growth is based on mean dry weight alone, and length is not used as a criterion of effect due to evidence that increased body depth and weight of healthy individuals is not adequately reflected in gains in body length during the test. A similar phenomenon could occur during the present 14-day *survival-and-growth* test using *H. azteca*.

Various statistical procedures can be used to assess the results of the test. The options, rationale for choice, and methods of calculation are discussed in depth in reports by the United States Environmental Protection Agency (1989; 1994a; b; c) and Environment Canada (1997d). The choice of statistical treatment depends on the test and study designs and, in particular, whether tests used replicate samples of sediment or multiple concentrations of test substances or materials. Sections 5 and 6 provide guidance on statistical endpoints and calculations.

4.8 Tests with Reference Toxicant(s)

The routine use of a reference toxicant or toxicants is necessary to assess, under standardized test conditions, the relative sensitivity of the culture(s) of *H. azteca*, and the precision and reliability of data produced by the laboratory. *Water only* tests with one or more reference toxicants are most commonly used in conjunction with *survival-and-growth* tests which measure sediment toxicity to *H. azteca* (see Appendix E). Procedures for spiking sediment with chemical(s) and for conducting *spiked sediment* reference toxicity tests are available or being developed (Burton, 1991; Smith *et al.*, 1992b; Suedel *et al.*, 1993a; b; EC, 1995) and should see wider use in the future. A static, 96-h *water only* reference toxicity test is recommended here for routine use with sediment toxicity tests using *H. azteca*, a practice followed by USEPA (1994a). This reference toxicity test may be supplemented or replaced with one or more *spiked sediment* tests with reference toxicant(s) after suitable procedures are standardized. Environment Canada's guidance document on using control sediment spiked with a reference toxicant should be consulted (EC, 1995).

Table 3 provides a checklist of conditions and procedures recommended for conducting static, 96-h *water only* reference toxicity tests using *H. azteca*. The recommended test procedure, which is largely consistent with USEPA (1994a), uses 2- to 9-day old amphipods to start the test. There are ten individuals per test chamber, at least five test concentrations plus a control (i.e., using control/dilution water only), and one or more replicates per treatment. Recommended test chambers are 300-mL, high form glass beakers or glass jars with an inner diameter of ~7 cm, and the recommended test volume is 200 mL solution/chamber. Solutions in test chambers are not aerated during the test and are normally covered to minimize contamination and losses due to evaporation. It is recommended that 0.5 mL YCT (or equivalent food; see Section 4.4) be added to each test chamber (including the controls) on Days 0 and 2 of the test.

Temperature and lighting conditions for this test procedure are the same as those described for definitive sediment toxicity tests (see Section 4.2 and Tables 2 and 3). Daily observations are made for numbers of dead or moribund amphipods in each test chamber. Temperature and dissolved oxygen are measured daily for each treatment; and pH, alkalinity, hardness, and conductivity are measured for each treatment at the start and end of the test (Section 4.5). The test

Table 3 Checklist of Recommended Conditions and Procedures for Conducting *Water Only* Reference Toxicity Tests using *Hyalella azteca*

Test type	– static 96-h *water only* toxicity test
Reference toxicant	– copper sulphate ($CuSO_4$), chloride ($CdCl_2$), potassium chloride (KCl), or sodium chloride (NaCl)
Frequency of test	– once/month, or in conjunction with definitive test(s) with sediment samples
Test solutions	– control and at least five test concentrations
Solution replacement	– none
Control/dilution water	– culture water or other clean ground or surface water; reconstituted fresh water if a high degree of standardization is required; natural or reconstituted seawater with salinity ≤15 ‰ for tests with estuarine sediment; DO, 90 to 100% saturation when used in test
Amphipods	– removed from known-age culture as <1- to 7-d olds and held in beaker for 2 d preceding test while fed; 2- to 9-d old at start of test; 10/test chamber
Substrate for amphipods	– one 2.5 × 2.5 cm strip of medicinal gauze bandage, presoaked in culture water for 24 h; or a 2.5 × 2.5 cm piece of Nitex™ or plastic mesh
Test chamber	– 300-mL high form glass beaker or glass jar, ~7 cm I.D.; normally covered
Volume of test solution	– 200 mL
Number of replicates	– one or more per concentration
Temperature	– daily average, 23 ± 1°C; instantaneous, 23 ± 3°C
Lighting	– overhead full-spectrum (fluorescent or equivalent); 500 to 1000 lux; 16-h light:8-h dark
Aeration	– none
Feeding	– YCT, 0.5 mL to each chamber on Days 0 and 2
Observations	– daily, each chamber, for number of dead or moribund amphipods
Measurements of water quality	– daily, each treatment, for DO and temperature; start and end of test, each treatment, for pH, alkalinity, hardness, conductivity
Endpoints	– mean percent survival, each treatment; 96-h LC50
Test validity	– results for reference toxicity test considered invalid if mean 96-h survival in control water <90%

endpoints are the mean percent survival in each treatment, and the 96-h LC50. Results for a reference toxicity test must be declared invalid if the mean survival in control water is <90% at the end of the test (Table 3).

Appropriate criteria for selecting suitable reference toxicants might include the following (EC, 1990; 1995):

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- can be interspersed evenly throughout clean substrate;
- good dose/response curve for test organism;
- stable in aqueous solution;
- minimal hazard posed to user;
- concentration easily analyzed with precision;
- known influence of water quality (e.g., pH, hardness) on toxicity of chemical to test organism; and
- known influence of physicochemical characteristics of sediment (e.g., particle size, organic carbon content) on toxicity of chemical to test organism.

Reagent-grade copper sulphate, cadmium chloride, potassium chloride, or sodium chloride are recommended for use with *H. azteca* as reference toxicants (USEPA, 1994a).

Reference toxicity tests using *H. azteca* and one or more of these chemicals must be performed monthly with the laboratory's established cultures (USEPA, 1994a). The performance of any cultures recently established in the laboratory using new breeding stock should also be evaluated using reference toxicant(s) before these cultures are used to provide test organisms (see Sections 2.3.1 and 2.3.11).

Pertinent reports by Environment Canada provide guidance on the selection, performance, and use of *water only* (EC, 1990) or *spiked-sediment* (EC, 1995) reference toxicity tests. Laboratory personnel unfamiliar with such tests are advised to consult these reports before preparing for or conducting them.

It is the laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicant(s) before conducting definitive sediment assays with *H. azteca*. To meet this responsibility, the laboratory personnel should initially determine intralaboratory precision, expressed as coefficient of variation (CV), by performing five or more tests with the reference toxicant(s) using different groups of *H. azteca* from separate known age cultures (Section 2.3.10) reared in the laboratory (USEPA, 1994a). For these preliminary tests, the same reference toxicant(s), concentrations, type/source of test water, and test procedure (i.e., Table 3) should be used. Performance of the routine (monthly) tests with reference toxicant(s) should continue to follow this same procedure. A series of test concentrations should be chosen[30], based on preliminary tests, to provide partial mortalities in two or more concentrations and enable calculation of a 96-h LC50 with acceptably narrow confidence limits (see Section 6.5).

Once sufficient data are available (EC, 1990; 1995) LC50s for a particular reference toxicant must be plotted successively on a warning chart, and examined to determine whether the results are within ± 2 SD of values obtained in previous tests with *H. azteca* using the same reference toxicant and test procedure. A separate warning chart must be prepared and updated for each reference toxicant used. The warning chart should plot logarithm of concentration on the vertical axis against date of the test or test number on the horizontal axis. Each new LC50 for the reference toxicant should be compared with established limits of the chart; the LC50 is acceptable if it falls within the warning limits.

The logarithm of concentration (including LC50) should be used in all calculations of mean and standard deviation, and in all plotting procedures. This simply represents continued adherence to the assumption by which each LC50 was estimated based on logarithms of concentrations. The warning chart may be constructed by plotting the logarithmic values of the mean and ±2 SD on arithmetic paper, or by converting them to arithmetic values and plotting those on the logarithmic scale of semi-log paper. If it were demonstrated that the LC50s failed to fit a log-normal distribution, an arithmetic mean and SD might prove more suitable. The mean of the available values of log(LC50), together with the upper and lower warning limits (±2 SD), should be recalculated with each successive LC50 for the reference toxicant until the statistics stabilize (EC, 1990; 1995; 1997d). If a particular LC50 fell outside the warning limits, the sensitivity of the test organisms and the performance and precision of the test would be suspect. Since this might occur 5% of the time due to chance alone, an outlying LC50 would not necessarily indicate abnormal sensitivity of the culture or unsatisfactory precision of toxicity data. Rather, it would provide a warning that there might be a problem. A thorough check of all culturing and test conditions and procedures should be carried out. Depending on the findings, it might be necessary to repeat the reference toxicity test, to obtain new breeding stock, and/or to establish new *known age* cultures, before undertaking further sediment toxicity tests.

Results that remained within the warning limits might not necessarily indicate that a laboratory was generating consistent results. Extremely variable data for a reference toxicant would produce wide warning limits; a new data point could be within the warning limits but still represent undesirable variation in test results. A coefficient of variation of no more than 30%, and preferably 20% or less, is suggested as a reasonable limit by Environment Canada (1990).

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[30] See Appendix H for guidance in selecting an appropriate series of test concentrations. Each successive concentration chosen should be at least 50% of the previous concentration.

Specific Procedures for Testing Field-collected Sediment or Similar Particulate Material

This section gives particular instructions for preparing and testing samples of field-collected sediment or similar particulate material. These instructions are in addition to the procedures listed in Section 4. Toxicity tests with these samples should be conducted using either the daily-renewal test option or the static test option (see Section 4), depending on the test objectives or constraints and on any related regulatory guidelines or requirements.

Detailed guidance for the collection, handling, transport, storage, and analyses of field-collected sediment is given in ASTM (1991b; 1995b) and Environment Canada (1994) reports specific to these subjects. Environment Canada (1994) should be consulted and followed (in addition to the guidance provided here), when collecting samples of field-collected sediment and preparing them for toxicity tests with *H. azteca*.

5.1 Sample Collection

Environment Canada (1994) provides a useful summary of field-sampling design and appropriate techniques for sample collection. Field surveys of sediment toxicity using biological tests with *H. azteca* and/or other suitable, sediment associated test organisms are frequently part of more comprehensive surveys. Such surveys could include a battery of toxicity tests to evaluate the toxicity of whole sediment, pore water, or elutriate, together with tests for bioaccumulation of contaminants, chemical analyses, biological surveys of epifaunal and/or infaunal organisms, and perhaps the compilation of geological and hydrographic data. Statistical correlation can be improved and costs reduced if the samples are taken concurrently for these tests, analyses, and data acquisitions.

Samples of sediment collected for assessment of an adverse effect on survival and growth of *H. azteca* might be routinely taken (e.g., quarterly, semi-annually, or annually) from a number of sites for monitoring and compliance purposes, or might be collected on one or more occasions during field surveys of sites for spatial (i.e., horizontal or vertical) or temporal definition of sediment quality. One or more sites should be sampled for reference (presumably clean) sediment during each field collection.[31]

The number of stations to be sampled at a study site and the number of replicate samples per station will be specific to each study. This will involve, in most cases, a compromise between logistical and practical constraints (e.g., time and cost) and statistical considerations. Environment Canada (1994) should be consulted for guidance with respect to the sampling design, including the recommended minimum number of field replicates.

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[31] A reference sediment is that collected near the site(s) of concern. Ideally, it possesses geochemical characteristics similar to those of the test sediment but without anthropogenic contaminants. It is not unusual for nearby reference sites to have some degree of contamination due to anthropogenic chemicals. In some instances, reference sediment might be toxic due to naturally occurring physical, chemical, or biological properties (Burton, 1991).

For certain monitoring and regulatory purposes, multiple replicates (i.e., separate samples from different grabs or cores taken at the same site) should be taken at each sampling station, including one or more reference stations (EC, 1992a; 1994; 1997a; USEPA, 1994a). Each of these field replicates must be tested for its toxicity to *H. azteca*, using five or more test chambers per replicate sample. The use of *power analysis* statistics (see Section 5.6) with endpoint data obtained in previous *survival-and-growth* tests at the same or similar sites will assist in determining if more than five laboratory replicates need to be tested with each field replicate. Also, some of the statistical tests have requirements for a minimum number of replicates. For certain other purposes (e.g., preliminary or extensive surveys of the spatial distribution of toxicity), the survey design might include only one sample from each station, in which case the sample would normally be homogenized and split between five or more test chambers (i.e., laboratory replicates). The latter approach precludes any determination of mean toxicity at a given sampling location (station), but allows a statistical comparison of toxicity of each sample with the control, and also if desired, a comparison among the test samples (stations), using appropriate statistical tests Sites for collecting reference sediment should be sought where the geochemical properties of the sediment are similar to sediment characteristics encountered at the test sites. Close matching of sediment grain size or organic content might not be necessary for this test, since *H. azteca* can tolerate uncontaminated sediments differing in these properties without changes in survival or growth (Section 1.4). Matching of organic carbon content might not be warranted in cases where pollution (e.g., from pulp mills or sewage) is responsible for the high organic content of test sediments. Preliminary surveys to assess the toxicity and geochemical properties of sediment within the region(s) of concern and at neighbouring sites are useful for selecting appropriate sites at which to collect reference sediment.

Samples of municipal or industrial sludge (e.g., sewage sludge, dewatered mine tailings, sludge from an industrial clarifier or settling pond) might be collected for assessment of their adverse effect on survival and growth of *H. azteca*, and for geochemical and contaminant analyses. Other particulate wastes (e.g., drilling mud residue) might also be taken for toxicity and chemical evaluation.

Procedures used for sample collection (i.e., core, grab, dredge, or composite) will depend on the study objectives and the nature of the sediment or other particulate material being collected. The types of sediment collection devices and their advantages and disadvantages have been summarized by Environment Canada (1994), and further details are provided elsewhere (de Groot and Zschuppe, 1981; Baudo *et al.*, 1990; Burton, 1992; Sly and Christie, 1992; ASTM, 1995b).

A benthic grab or core rather than a dredge should be used for sampling sediment, to minimize disruption of the sample. Sediment to be evaluated for toxicity and chemistry should be collected from one or more depths that represent the layer(s) of concern (e.g., a surficial 2-cm layer, or a deeper layer if there are concerns about historical deposition of contaminants).

Care must be taken to minimize loss of fines during sample collection. If the sample is obtained using a grab sampler, hand corers should be used to collect a sample from the surficial 2 cm, or desired layer, of the test sediment retrieved using this sampling device. This can be achieved if the grab can be opened from the top to expose the surface of the undisturbed sediment. The sample should be transferred to a clean sample container.

Before commencing a sampling program, the required volume of sediment per sample should be calculated (EC, 1994). This calculation should take into account the quantity of sediment required to prepare laboratory replicates for sediment toxicity tests, as well as that required for particle size characterization, percent organic matter, percent moisture, and specific chemical analyses. A volume of at least 5 to 7 L of sediment per sample is normally required (EC, 1994); although this will depend on the study objectives/design and the nature of the chemical analyses to be performed. To obtain the required sample volume, it is frequently necessary to combine subsamples retrieved using the sampling device. Guidance provided in Environment Canada (1994) for compositing subsamples in the field should be followed.

The same collection procedure should be used at all field sites sampled. Environment Canada (1994) should be consulted for further guidance on appropriate devices and procedures for sample collection.

5.2 Sample Labelling, Transport, Storage, and Analyses

Containers for transport and storage of samples of field-collected sediment or similar particulate material must be made of nontoxic material. The choice of container for transporting and storing samples depends on both sample volume and the potential end uses of the sample. Environment Canada (1994) should be consulted for guidance in selecting suitable containers. The containers must either be new or thoroughly cleaned, and rinsed with test water or other clean water (e.g., distilled or deionized water) before use.

Each sample container should be filled completely, to exclude air. Immediately after filling, each sample container must be sealed, and labelled or coded. Labelling and accompanying records made at this time must include at least a code or description which identifies sample type (e.g., grab, core, composite), source, precise location (i.e., water body, latitude, longitude, depth), replicate number, and date of collection; and should include the name and signature of sampler(s). Persons collecting samples of sediment should also keep records describing details of:

- the nature, appearance, and volume of each sample;

- the sampling procedure and apparatus;

- any procedure used to composite or subsample grabs or cores in the field;
- the number of replicate samples taken at each sampling station;
- the sampling schedule;
- the types and numbers of containers used for transporting samples;
- any field measurements (e.g., temperature, salinity, pH, dissolved oxygen) of the overlying water or sediment at the collection site; and
- procedures and conditions for cooling and transporting the samples.

Upon collection, warm (>7°C) samples should be cooled to between 1 and 7°C with regular ice or frozen gel packs, and kept cool (4 ± 3°C) in darkness throughout the period of transport. As necessary, gel packs, regular ice, or other means of refrigeration should be used to assure that the temperature of the sample(s) remains within 1 to 7°C during transit.

The date of receipt of the sample(s) at the laboratory must be recorded. Sample temperature upon receipt at the laboratory should also be measured and recorded. Samples to be stored for future use must be held in airtight containers and in darkness at 4 ± 2°C (EC, 1994; 1997a). Any air "headspace" in the storage container should be purged with nitrogen gas, before capping tightly (EC, 1994). Samples must not freeze or partially freeze during transport or storage, and must not be allowed to dry (EC, 1992a; 1994; 1997a). It is recommended that samples of sediment or similar particulate material be tested as soon as possible after collection. The sediment toxicity test should begin within two weeks of sampling, and preferably within one week; the test must start no later than six weeks after sample collection.[32]

Ideally, sediment characteristics that are unstable (e.g., pH, oxidation-reduction potential) or changed by conditions of transit and storage (e.g., temperature) should be measured in the field to help characterize the sample. In the laboratory, each sample of field-collected sediment should be thoroughly mixed (Section 5.3), and representative subsamples taken for physicochemical characterization. Each sample (including all samples of control and reference sediment) must be characterized by analyzing subsamples for at least the following (USEPA, 1994a): for whole sediment — particle size distribution (percentage of coarse-grained sand, medium-grained sand, fine-grained sand, silt, and clay), percent water content, and total organic carbon content; for pore water — pH and ammonia (total and un-ionized concentrations; see Section 4.5). Other analyses could include (USEPA, 1994a; APHA *et al.*, 1995): total inorganic carbon, total volatile solids, biochemical oxygen demand, chemical oxygen demand, cation exchange capacity, acid volatile sulphides, metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons, and porewater analyses for various physicochemical characteristics. Unless indicated otherwise, identical chemical, physical, and toxicological analyses should be performed with subsamples representative of each replicate sample of field-collected sediment (including reference sediment) taken for a particular survey of sediment quality, together with one or more subsamples of control sediment.

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[32] The toxicity and geochemistry of contaminated sediments from Hamilton Harbour was reported to change with storage for longer than one week, although the data supporting that statement were not provided (Brouwer *et al.*, 1990). A study by Othoudt *et al.* (1991) found that the toxicity of samples of freshwater sediment did not differ significantly when stored at 4°C for periods of 7 to 112 days. Burton (1991) and USEPA (1994a) report studies by various researchers showing in some instances that the toxicity of sediment held at 4°C was unchanged after several months' storage, and in other cases that changes were noted within days to weeks. A recommendation for testing within two weeks conforms with the advice in other sediment toxicity tests by Environment Canada (1992a; 1997a). A maximum permissible storage time of six weeks has been recommended by Environment Canada (1994) for sediments intended for toxicity tests, in view of practical difficulties for shorter times, including time required if initial chemical analyses are to be performed.

5.3 *Preparing Sample for Testing*

Field-collected sediment or similar particulate waste material should normally not be prepared for testing by sieving with water, as this would remove contaminants present in the pore water or loosely sorbed to particulate material (EC, 1994). Large debris or large indigenous macro-organisms should normally be removed using forceps or a gloved hand.

The presence of indigenous macro-organisms in samples can reduce the growth of *H. azteca* in freshwater sediment toxicity tests, and can confound the interpretation of test results (Reynoldson *et al.*, 1994). If a field-collected sediment contains a large number of indigenous macro-organisms which cannot be removed using forceps, the sample may be press-sieved (not washed) through one or more suitably sized mesh screens. For those sediments containing small macro-organisms which, due to sediment characteristics cannot be removed by press-sieving, the sample(s) may be rinsed through a fine-mesh sieve (e.g., 0.25 to 0.5 mm; Day *et al.*, 1995b) using any liquid that has separated from the sample during its transport and/or storage. This liquid must be remixed within the sieved sample (Section 4.1).

Sieving could alter the concentration or bioavailability of contaminants in the sediment, or alter its nutrient content and/or particle size (EC, 1994; Day *et al.*, 1995b). If sediments are sieved, therefore, it is recommended that the physicochemical properties of the sediment (e.g., porewater metals, particle size distribution) be documented before and after sieving. Comparative toxicity tests using sieved and unsieved sediment might, in some cases, also be necessary or appropriate to discern the effect of sieving on sample toxicity.

Unless research or special study objectives dictate otherwise, each sample of field-collected test material should be homogenized in the laboratory before use (EC, 1994; USEPA, 1994a).[33] Mixing can affect the concentration and bioavailability of contaminants in the sediment, and sample homogenization might not be desirable for all purposes.

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[33] One of the reasons for routinely homogenizing samples is to mix into the sediment, any pore water which rises to the surface during sample shipment and storage. Homogenization is also necessary to redistribute the sample constituents that have compacted and layered according to particle size during transport and storage.

To achieve a homogeneous sample, either mix it in its transfer/storage container, or transfer it to a clean mixing container. The sample may be stirred using a nontoxic device (e.g., stainless steel spoon or spatula), until its texture and colour are homogeneous (EC, 1992a). Alternatively, a mechanical method (USEPA, 1994a; EC, 1994) may be used to homogenize the sample. For each sample included in a test, mixing conditions including duration and temperature must be as similar as possible. If there is concern about the effectiveness of sample mixing, subsamples of the sediment should be taken after mixing, and analyzed separately to determine homogeneity.

Immediately following sample mixing, subsamples of test material required for the toxicity test and for physicochemical analyses must be removed and placed in labelled test chambers (Section 4.1), and in the labelled containers required for storage of samples for subsequent physicochemical analyses. Any remaining portions of the homogenized sample that might be required for additional toxicity tests using *H. azteca* or other test organisms should also be transferred to labelled containers at this time. All subsamples to be stored should be held in sealed containers with no air space, and must be stored in darkness at 4 ± 2°C (Section 5.2) until used or analyzed. Just before it is analyzed or used in the toxicity test, each subsample must be thoroughly remixed to ensure that it is homogeneous.

5.4 Test Water

For tests with field-collected sediment or similar particulate material, the water introduced to test chambers (i.e., overlying water) may be from the same source as that used for culturing *H. azteca* (see Sections 2.3.4 and 3.4). Alternatively, this water may be from a separate supply of natural fresh or estuarine water, or reconstituted water. For certain applications, the experimental design might require or endorse the use of fresh or estuarine water taken from the reference site nearby where test sediments were collected. Use of uncontaminated site water, or uncontaminated water adjusted to the hardness of site water, is frequently a good choice due to the modifying influence of waters with different hardness values on the toxicity of metals or organic contaminants in sediment. Section 2.3.4 provides pertinent guidance on the preparation and analysis of water to be used as overlying water in the test.

5.5 Test Observations and Measurements

A qualitative description of each field-collected test material should be made when the test is being set up. This might include observations of sample colour, texture, and homogeneity; and the presence of plants, animals, and tracks or burrows of animals (EC, 1992a). Any changes in the appearance of the test material and in the overlying water, observed during the test or upon its termination, should be noted and reported.

Measurements of the quality of the overlying water (e.g., pH, temperature, hardness, alkalinity, ammonia, dissolved oxygen content) in test chambers should be made during or at the beginning and end of the test, as described in Section 4.5. Depending on the test objectives and experimental design, separate test chambers might also be set up at the beginning of the test (Section 4.1), to monitor whole sediment and/or porewater chemistry (USEPA, 1994a). These would be destructively sampled during and at the end of the test. Test organisms might or might not be added to these extra test chambers, depending on study objectives. Measurements of chemical concentrations in the sediment or pore water within these chambers may be made by siphoning most of the overlying water without disturbing the surface of the sediment, then removing aliquots of the sediment for the appropriate analyses (see Section 5.2). If pore water were to be analyzed, centrifugation without filtration would be the recommended sampling procedure (EC, 1994; USEPA, 1994a). Environment Canada (1994) should be consulted for guidance on the recommended procedure for extracting pore water, and its treatment and storage before analyses.

Depending on the study objectives and the nature of the test sediments (e.g., rich in organics), measurements of porewater pH and ammonia concentrations might be made as the test progresses, using test chambers dedicated for this purpose (EC, 1994; USEPA, 1994a). Other sediment characteristics (e.g., concentrations of metals, hydrogen sulphide, total volatile solids, Eh) might be monitored in the same test chambers. If it were desired to monitor these variables, at least one chamber should be set up for each treatment, and destructively sampled for this purpose.

5.6 *Test Endpoints and Calculations*

The common theme for interpreting tests with samples of sediment, is a comparison of the biological effects with the effects found in a reference sediment. The reference sample should be used for comparative purposes whenever possible or appropriate, because this provides a site-specific evaluation of toxicity (USEPA, 1994a). Sometimes the reference sediment might be unsuitable for comparison because of toxicity or atypical physicochemical characteristics. In such cases, it would be necessary to compare the test sediments with the control sediment. Control sediment(s) results will assist in distinguishing contaminant effects from noncontaminant effects caused by such things as particle size and organic carbon content. Regardless of whether the reference sediment or control sediment is used for the statistical comparisons, the results from control sediment must be used to judge the validity and acceptability of the test (Section 4.2).

Analysis of results will differ according to the purposes and particular designs of the test. This section covers the analytical procedures, starting with the simplest design and proceeding to the more complex designs. Standard statistical procedures are generally all that is needed for analyzing the results. Investigators should consult Environment Canada (1997d) as well as USEPA (1994a;, Section 14) and USEPA/USACE (1994; Appendix D) for guidance on the appropriate statistical endpoints and their calculation. As always, advice of a statistician familiar with toxicology should be sought for design and analysis of tests.

Analysis of variance (ANOVA) and multiple-comparison tests are commonly used for statistical interpretation of sediment tests. This is an hypothesis-testing approach, and is subject to the appreciable weaknesses described in Section 6.5.3. Notably, any increased variability within the test will weaken its power to distinguish toxic effects, resulting in an endpoint at a higher concentration (i.e., less toxicity is concluded). Similarly, use of only a few replicates instead of many replicates will weaken the discrimination of a test and will lead to a conclusion of less apparent toxicity, other things being equal (see Section 5.6.2). There is no alternative to hypothesis-testing for analysis of most toxicity tests with sediment, because they use only one concentration of samples, usually full-strength. There are superior alternatives for point estimates of toxicity if various concentrations are tested (see Section 6).

The parametric analyses with ANOVA and multiple-comparison tests assume that the data are normally distributed, and that the variance is homogeneous among the different groups. As the first step in analysis, these assumptions should be tested with *Shapiro-Wilk's Test* for normality and *Bartlett's Test for Homogeneity of Variance* (Eisenhart *et al.*, 1947; Sokal and Rohlf, 1969). If the data are satisfactory by these tests, analysis may proceed. If not, data could be transformed (e.g., as square roots, logarithms, or as arcsine square root for quantal data which are to be used in quantitative analysis; Mearns *et al.*, 1986). The tests for normality and homogeneity might then show conformance with normality and homogeneity, and in fact that is a likely outcome of transformation.

Parametric tests are actually quite robust in the face of moderate deviations from normality and equality of variance. Therefore, parametric analysis (e.g., ANOVA and multiple comparison) should proceed, even if moderate nonconformity continues after transformation. Excluding a data-set for minor irregularities might lose a satisfactory and sensitive analysis and forgo the detection of real effects of toxicity.[34] Analysis by nonparametric tests should also proceed in parallel, with the more sensitive (lower endpoint) of the two analyses providing the final estimates of toxicity (see further explanation and rationale in Environment Canada, 1997d).

Multi-concentration tests might be conducted with sediment, sludge, or similar particulate material. Measured amounts of the test sample could be mixed with measured quantities of natural or formulated control sediment (see Sections 3.5 and 6.2). Procedures for mixing different samples of sediment are not yet standardized or proven (see Section 6.2), and caution is advised due to possible nonlinear responses and changes in bioavailability or sorption characteristics (Nelson *et al.*, 1994). A minimum of five subsamples are recommended to provide replicates in each concentration, to determine sample homogeneity and test precision. Statistical analyses to determine endpoints are described in Section 6.5.

5.6.1 Variations in Design and Analysis

A very preliminary survey might have only one test sample and one reference sample, without replication. Simple inspection of the results might provide guidance for designing more extensive studies.

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[34] Tests for normality and homogeneity become less meaningful with the small samples of environmental toxicology. Plotting and examining the general nature of the distribution and apparent deviations can be more revealing and is recommended (EC, 1997d). Equality in sample sizes and the magnitude of variation are probably more important factors for the outcome of parametric analysis, but they have received scant attention in toxicology. Robustness of ANOVA is shown by its ability to produce realistic probabilities if the distribution of data is reasonably symmetrical, and if treatment variances are within threefold of each other (Newman, 1995).

If there were a single test sample and a reference sample, with equal replication for each, a standard *Student's t-test* would be suitable for analysis (Paine and McPherson, 1991a). The t-test is fairly robust. The full formula for the t-test should be used, because it contains provisions for irregular data. The formula handles unequal numbers of replicates in the test and reference samples, as well as unequal variances in the two groups (USEPA/USACE, 1994). If there were *laboratory replicates* only, the interpretation of findings would be different from a survey in which there were *field replicates*, as described in the following for analysis of variance.

A preliminary study might conceivably be run with samples from many stations, but without either field replicates or laboratory (within-sample) replicates. The objective might be to identify a reduced number of sampling stations deserving of more detailed and further study. Opportunities for statistical analysis would be limited. The nonreplicated test data could be compared with the reference data using outlier detection methods (USEPA, 1994a; Newman, 1995; EC, 1997d). A sample would be considered toxic if its result was rejected as an extreme value when considered as part of the reference and/or control data.

A more usual survey of sediments would involve the collection of samples from several places using the same methods, and their comparison with a single reference and/or control. There are several pathways for analysis, depending on the type and quality of data, but often there would be an *analysis of variance (ANOVA)* followed by one of the multiple-comparison tests. In the ANOVA, the reference would also be treated as a "location".

In these multi-location surveys, the type of replication would make a difference in how the results were interpreted. There might be one sample at each of a number of locations, with *laboratory replicates* obtained by subdividing each sample. In that case, the one-way ANOVA would distinguish whether there was an overall difference among locations, a difference that was greater than the baseline variability in the *within-laboratory* procedures for setting up and running the test. Sampling variability would not really be assessed in the statistical analysis, except that it would contribute to any difference found in locations. If *field replicates* were collected at each of the sampling locations, and no laboratory replicates were used, the same type of one-way ANOVA would evaluate overall difference in locations, over and above the *combined* variability of sampling the location and running the test. It would be unusual but much more powerful, to have field replicates for all sampling locations, and also laboratory replicates of each field replicate. If that were done, the laboratory replicates would become the replicates in a nested one-way ANOVA, and would be the base of variability for comparing differences in the samples. The ANOVA could be used to see (a) if there was an overall difference in locations, and (b) whether there was an overall difference in replicates taken at the various locations. After an ANOVA, the analysis would proceed to one or more type of multiple-comparison test, as described in the following.

After the ANOVA for multi-location surveys, different statistical tests are available for application. If it were desired to compare each sampling location with the reference to see if the two were different, *Dunnett's test* should be used. It assumes normality and equal variance, and is based on an *experiment-wise* value of α (the probability of declaring a significant difference when none actually exists). If replication were unequal, investigators should seek the advice of a statistician, or adopt the complete interlocation comparison described in the following paragraph.

In a multi-location survey, an investigator might wish to know which sampling locations were different from which others, as well as knowing which ones were different from the reference and/or control. Such a situation might involve a number of locations "downstream" of an effluent discharge and one might want to know which locations had significantly higher toxicity than others, and were particularly deserving of cleanup. *Tukey's test* is designed for such an analysis, and is commonly found in statistics packages. It can deal with unequal sample sizes.[35]

If it were desired to compare each sampling location with the reference, but the data did not conform to requirements of normality and equal variance, the ANOVA and subsequent tests would be replaced by nonparametric tests. *Steel's Many-One Rank test* would be used if replication were equal, while unequal replication would require *Wilcoxon Rank Sum test with Bonferroni's adjustment.*

5.6.2 Power Analysis

An important factor to consider in the analysis of toxicity tests with sediment is the potential for declaring false "positives" (i.e., calling a clean site dirty) or false "negatives" (i.e., calling a dirty site clean). Scientists are usually cautious in choosing the level of significance (α) for tolerating false positive results (Type I error), and usually set it at P = 0.05 or 0.01. Recently, toxicologists have been urged to report both α and statistical power ($1 - \beta$), i.e., the probability of correctly rejecting the null hypothesis (H_0) and not making a Type II error. There are several factors that influence statistical power, including:

- variability of replicate samples representing the same treatment;

- α (i.e., the probability of making a Type I error);

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[35] An alternative approach is currently recommended for sediment testing in the United States. (USEPA, 1994a; USEPA/USACE, 1994). For equal replicates, *Fisher's Least Significant Difference (LSD)* is said to be a superior approach. It is based on a smaller "pairwise error rate" for α in comparing any given location with another, but holds the overall value of α to the pre-selected value (usually 0.05). *LSD* is seldom included in software packages for toxicity, but it is described in some textbooks (e.g., Steel and Torrie, 1980), and is detailed in Appendix D of USEPA/USACE (1994). *Tukey's test* is instead recommended here, partly because *LSD* might declare significant differences too readily. *LSD* is also intended for only a few of all the possible comparisons in a set of data, and those comparisons would have to be specified in advance. In the current U.S. recommendations for pairwise comparison, a situation with unequal replicates would call for *Bonferroni's adjustment of the t-test* to replace testing by *LSD* (USEPA, 1994a). That adjusted t-test enables all possible comparisons among locations.

- effect size (*ES*) (i.e., the magnitude of the true effect for which you are testing); and
- *n* (i.e., the number of samples or replicates used in a test).

Power analysis can be used *a priori* to determine the magnitude of the Type II error and the probability of false positive results. It can also be used to ascertain the appropriate number of field and laboratory replicates for subsequent surveys involving this test, or to assist in the selection of future sampling sites. It is always prudent to include as many replicates in the test design as is economically and logistically warranted (see Section 5.1); power analysis will assist in this determination. A good explanation of the power of a test, and how to assess it, can be found in USEPA (1994a), with some coverage in EC (1997d).

Section 6

Specific Procedures for Testing Spiked Sediment

This section gives guidance and instructions for preparing and testing control or other sediment spiked experimentally with chemical(s), contaminated sediment, or complex waste mixtures. These recommendations and instructions are in addition to the procedures listed in Section 4. More detailed and appropriate guidance for spiking sediment with chemical(s) and conducting toxicity tests with chemical/sediment mixtures is given in Environment Canada (1995). Depending on the test objectives or constraints, and on any related regulatory guidelines or requirements, these toxicity tests can be conducted using either the daily-renewal test option or the static test option (see Section 4). Further evaluation and standardization of procedures for spiking sediment (Section 6.2) might be required before sediment toxicity tests using *H. azteca* or other appropriate test organisms are applied to evaluate specific chemical/sediment mixtures for regulatory purposes.

The cause(s) of sediment toxicity and the interactive toxic effects of chemical(s), contaminated sediment, or particulate waste in association with otherwise clean sediment can be examined experimentally by spiking *clean* control sediment with these substances or materials. The spiking might be done with one or more chemicals, with another sediment (clean or uncontaminated), or with similar particulate material (e.g., dredged sludge mixed with sediment from an existing or prospective freshwater disposal site).

Toxicity tests using sediment spiked with a range of concentrations can estimate LC50s, and can determine concentrations causing sublethal effects. The influence of the physicochemical characteristics of natural or formulated sediment on chemical toxicity can also be determined with spiked-sediment toxicity tests. Reference toxicity tests can also be conducted using control sediment spiked with an appropriate chemical (see Section 4.8). Specific recommendations and instructions for performing spiked-sediment tests are provided in this section. (Additional useful guidance is given in USEPA, 1994a and EC, 1995.)

6.1 Sample Properties, Labelling, and Storage

Information should be obtained on the properties of the chemical, contaminated sediment, or particulate waste to be diluted experimentally with control or other sediment. For samples of contaminated sediment or similar particulate material, instructions on sample characterization (Section 5.2), should be followed. For individual chemicals, chemical substances (e.g., formulated products), or chemical mixtures, available information should be obtained on the concentration of major ingredients and impurities, water solubility, vapour pressure, chemical stability, dissociation constants, toxicity to humans and aquatic organisms, and biodegradability. Where aqueous solubility is in doubt or problematic, acceptable procedures previously used for preparing aqueous solutions of the chemical(s) should be obtained and reported, and chemical solubility in test water should be determined experimentally. Other available information such as structural formulae, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient, should be obtained and recorded.

Chemical(s) to be tested should be at least reagent grade, unless a test on a formulated commercial product or technical grade chemical(s) is required (USEPA, 1994a). Chemical containers must be sealed and coded or labelled upon receipt. Required information (chemical name, supplier, date received, person responsible for testing, etc.) should be indicated on the label and/or recorded on a separate datasheet dedicated to the sample, as appropriate. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Sample(s) of contaminated sediment or particulate waste to be evaluated in spiked-sediment assays should be collected, labelled, transported, and stored according to instructions herein (Sections 5.1 and 5.2).

6.2 *Preparing Test Mixtures*

Different procedures have been used by researchers to spike (dose) *clean* control sediment with chemical(s), or to dilute contaminated sediment or other particulate waste with control sediment, in preparation for sediment toxicity tests with the mixture (ASTM, 1991a; b; 1993; 1995a; b; Burton, 1991; USEPA, 1994a; Hoke *et al.*, 1995). Mixing technique and time, as well as the period of aging after mixing, can affect the toxicity of the mixture (USEPA, 1994a).

Experimental procedures (including substance or material addition and mixing, equilibration time and conditions) used to prepare spiked sediment are new, varied, and not standardized. Accordingly, a standardized methodology for preparing spiked sediment cannot be recommended at this time. Rather, some of the approaches used previously or thought to be reasonable for preparing spiked sediment for toxicity tests with *H. azteca* are given here.

Environment Canada (1994; 1995) reports provide more detailed instructions and recommendations for spiking and homogenizing sediment, and should be consulted for further guidance. Researchers intending to pursue toxicity tests using one or more laboratory-prepared mixtures should proceed cautiously, and should be well aware of potential problems due to nonhomogeneity of the mixture(s) and the associated changes in bioavailability/sorption characteristics and nonlinear toxic responses that might result (Nelson *et al.*, 1994).

The method to be used for experimentally spiking sediment is contingent on the study objectives and the nature of the test substance or material to be mixed with control or other sediment. In many instances, a chemical/sediment mixture is prepared by making up a stock solution of the chemical and then mixing one or more measured volumes into control sediment (Swartz *et al.*, 1985b; 1988; ASTM, 1991a; 1993). Chemical concentrations in sediment are frequently calculated and expressed as µg/g or mg/kg dry weight (Swartz *et al.*, 1985b; 1988), although concentrations based on wet weight might be more useful for relating results to sediment toxicity (Burton, 1991). Depending on the nature of the test substance or material and test objectives, concentrations might also be normalized to sediment organic carbon content (e.g., for evaluating the toxicity of nonpolar organic compounds) or to acid volatile sulphides (e.g., for assessing metal toxicity) (Di Toro *et al.*, 1990; 1991; ASTM, 1991a; 1993; USEPA, 1994a).

The preferred solvent for preparing stock solutions is test water (see Sections 2.3.4 and 3.4); use of a solvent other than water should be avoided unless it is absolutely necessary. For organic compounds or other chemicals that do not dissolve readily in test water, a water-miscible organic solvent may be used to help disperse the compound in water (Borgmann *et al.*, 1990; ASTM, 1991a; 1993; USEPA, 1994a). Triethylene glycol has been recommended because of its low toxicity to aquatic organisms, low volatility, and high ability to dissolve many organic chemicals (ASTM, 1991a; 1993). Other solvents such as dimethylsulphoxide, methanol, ethanol, or acetone may be used to prepare stock solutions of organic chemicals, although they might contribute to sample toxicity, alter sediment properties, or be lost from the test material due to their volatility. Surfactants should not be used (ASTM, 1991a; 1993).

If an organic solvent is used, the test must be conducted using both a clean sediment control (i.e., no solvent and no test substance) and a sediment control containing solvent. For this purpose, a *solvent control* sediment must be prepared containing the concentration of solubilizing agent that is present in the highest concentration of the test chemical in sediment. Solvent from the same batch used to make the stock solution must be used (ASTM, 1991a; 1993; USEPA, 1994a).

Solvents should be used sparingly as they might contribute to the toxicity of the prepared test sediment. The maximum concentration of solvent in the sediment should be at a concentration that does not affect the survival or growth of *H. azteca* during the test. If this information is unknown, a preliminary *solvent only* test, using various concentrations of solvent in control sediment, should be conducted to determine the threshold-effect concentration of the particular solvent being considered for use in the definitive test.

Measured volumes of a stock solution containing test chemical(s) should be mixed with control (or other) sediment in a manner resulting in a homogeneous distribution of the chemical(s) throughout the sediment. Mixing may be by hand (e.g., using a clean spatula or glass rod), or by using a mechanical stirring or mixing device (e.g., Ditsworth *et al.*, 1990). Alternatively, the chemical can be coated on the walls of a flask and an aqueous slurry (i.e., control sediment and test water) added. The flask contents are then mixed by agitation. Another alternative is to add a measured volume of the stock chemical solution directly to a slurry of control (or other) sediment in test water, agitate the mixture, and allow it to settle (EC, 1992a). Other methods of mixing might prove to be acceptable provided that the chemical is shown to be evenly distributed in the sediment. Mixing conditions, including solution:sediment ratio, mixing and holding time, and mixing and holding temperature, must be standardized for each treatment included in a test. Time for mixing a spiked sediment should be adequate to ensure homogeneous distribution of the chemical, and may be from minutes up to 24 h. During mixing, temperature should be kept low to minimize changes in the mixture's physicochemical characteristics and microbial activity. Analyses of subsamples of the mixture are advisable to determine the degree of mixing and homogeneity (Ditsworth *et al.*, 1990; USEPA, 1994a).

For some studies, it might be necessary to prepare only one concentration of a particular mixture of control (or other) sediment and chemical(s), or a mixture of only one concentration of contaminated sediment or particulate waste in control or other sediment. For instance, a single-concentration test might be conducted to determine whether a specific concentration of chemical or particulate waste in clean sediment is toxic to *H. azteca*. Such an application could be used for research or regulatory purposes.

A multi-concentration test, using a range of concentrations of chemical added to a control or other sediment under standardized conditions, should be used to determine the endpoint (e.g., LC50, ICp, NOEC, LOEC; see Section 6.5) for chemical/sediment mixtures. A multi-concentration test using control sediment spiked with a specific particulate waste might also be appropriate. For such purposes, at least five test concentrations plus a control must be prepared; and the preparation and use of six to eight concentrations (plus one or more control sediments) is recommended to improve the likelihood of attaining each endpoint sought. An appropriate geometric dilution series may be used, in which each successive concentration of chemical or particulate waste in sediment is at least 50% of the previous (e.g., 10, 5, 2.5, 1.25, 0.63 mg/kg).[36] Test concentrations may also be selected from other appropriate logarithmic dilution series (see Appendix H). To select a suitable range of concentrations, a preliminary or range-finding test covering a broader range of test concentrations may be conducted.

Tests intended to evaluate the toxicity of mixtures of test substance(s) or material(s) in control sediment for federal registration or other regulatory purposes must be set up using a minimum of five replicates for each test concentration and each control sediment to be included in the assay. Since the objective for a multi-concentration test is to determine both LC50 (mortality data) and ICp (dry weight data), a test using six to eight concentrations plus control(s) is recommended. The number of replicates per treatment could be reduced or eliminated altogether for range-finding tests and, depending on the expected variance among test chambers within a treatment, could also be reduced or eliminated for nonregulatory screening assays or research studies.

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[36] Concentrations in sediment are normally calculated and expressed as µg/g or mg/kg, on a dry-weight or wet-weight basis. In some instances, concentrations in pore water might also be measured and expressed as µg/L or mg/L.

It is recommended that mixtures of spiked sediment be aged for four weeks before starting a test, in keeping with a common practice (USEPA, 1994a). Although many studies with spiked sediment have been started within a few hours or days of preparing the mixtures, such short and variable time periods might not be long enough for equilibration of the chemicals mixed in control sediment. A consistent four-week period of aging a mixture before initiating a toxicity test would provide some standardization for intra- and interlaboratory comparisons of results for tests with spiked sediment. Once prepared, each mixture should be placed in a suitable, sealed (with no air space) container, and stored in the dark at 4 ± 2°C (Section 5.2) for four weeks before use.

Based on the objectives of the test, it might be desirable to determine the effect of substrate characteristics (e.g., particle size or organic content) on the toxicity of chemical/sediment mixtures. For instance, the influence of sediment particle size on chemical toxicity could be measured by conducting concurrent multi-concentration tests with a series of mixtures comprised of the test chemical mixed in differing fractions (i.e., segregated particle sizes) or types of natural or formulated control sediment (Section 3.5). Similarly, the degree to which the organic content of sediment can modify chemical toxicity could be examined by performing concurrent multi-concentration tests using different chemical/sediment mixtures prepared with a series of organically enriched control sediments. Each fraction or formulation of natural or artificial control sediment used to prepare such mixtures should be included as a control in the test.

Tests could be required to measure the effect on survival and growth of *H. azteca* at test end, for one or more concentrations of specific chemicals introduced to the test chamber as a solution overlying the sediment. Procedures for preparing test concentrations could vary depending on the objectives of the study. One approach would be to carefully add the test solution(s) to replicate chambers containing a layer of control or other (e.g., field-collected) sediment, with no disturbance or subsequent mixing of the sediment and test solution(s). A second approach would require the test solution(s) introduced to test chambers to be agitated for a predetermined time period in the presence of the sediment before the test organisms are introduced. Chemical/sediment interactions might differ appreciably depending on the approach taken, and could result in a markedly different test result. Unless specified or otherwise required, test water adjusted to 23 ± 1°C should be used to prepare each test solution (Section 6.3). Replicate controls, including solvent controls if a solvent is used, must be prepared and treated identically. Instructions provided earlier in this section on the use of solvents other than water should be followed in preparing solvent controls.

6.3 Test and Control/Dilution Water

The water used for preparing stock or test solutions of chemicals and as test water in 14-day assays with mixtures of spiked sediment should normally be clean test water (see Section 3.4). The source of this water may be reconstituted water or natural water, and might or might not be identical to the water used for culturing the test organisms (see Section 2.3.4). Reconstituted water with a hardness of 90 to 100 mg $CaCO_3$/L (Section 2.3.4; USEPA, 1994a) is recommended if a high degree of standardization is required. For example, the use of a standard reconstituted water is recommended in instances where the measured toxicity of the chemical/sediment mixture is to be compared and assessed relative to toxicity data derived at a number of test facilities for this and/or other chemicals.

6.4 Test Observations and Measurements

A qualitative description of each mixture of spiked sediment and of the overlying test water should be made when the test is being established. This might include observations of the colour, texture, and visual homogeneity of each mixture of spiked sediment, and observations of the colour and opacity of the overlying water. Any change in appearance of the test mixture or overlying water noted during the test, or upon its termination, should be recorded. Measurements of the quality of each mixture of spiked sediment being tested (including the control sediment), and of the overlying water, should be made and recorded as described in Sections 4.5, 5.2, and 5.5.

If analytical capabilities permit, it is recommended that stock solutions, overlying water, sediment, pore water, and test solutions (if studied) be analyzed to determine the chemical concentrations, and to assess whether the sediment has been spiked satisfactorily. In instances where chemical concentrations are to be measured, sample aliquots should be taken from the high, medium, and low test concentrations at the beginning and end of the test, as a minimum. These should be preserved, stored, and analyzed according to suitable, validated procedures.

Unless there is good reason to believe that the chemical measurements are not accurate, toxicity results for any tests in which concentrations are measured should be calculated and expressed in terms of those average measured concentrations determined for both the whole sediment (μg/kg or mg/kg, dry weight) and the pore water (μg/L or mg/L). In cases where concentrations of chemical added to the overlying water are being tested, results should again be expressed as the average measured concentrations determined for the sediment and the pore water, although average chemical concentrations measured for the test solutions overlying sediment should also be calculated and reported (EC, 1992a).

6.5 Test Endpoints and Calculations

Multi-concentration tests with mixtures of spiked sediment are characterized by the 14-day LC50 and an endpoint representing the weight data (e.g., ICp). Appropriate statistics and programs for calculating these endpoints are summarized in this section. Section 5.6 provides guidance for calculating and comparing endpoints for single-concentration tests performed with mixtures of spiked sediment. For further information on the appropriate parametric or nonparametric statistics to apply to the endpoint data, the investigator should consult the Environment Canada report on statistics for the determination of toxicity endpoints (EC, 1997d), as well as USEPA (1994a; b; c) or USEPA/USACE (1994).

6.5.1 Median Lethal Concentration (LC50)

When a muti-concentration test with spiked sediment mixtures is conducted (Section 6.2), the quantal mortality data must be used to calculate the 14-day *median lethal concentration (LC50)*, together with its 95% confidence limits. To estimate an LC50, mortality data at 14 days are combined for all replicates at each concentration. If mortality is not ≥50% in at least one concentration, the LC50 cannot be estimated. If there are no mortalities at a specific concentration, that information is used as an effect of 0% mortality. However, if successive concentrations yield a series of 0% mortalities, only the highest concentration of the series should be used in estimating the LC50 (i.e., the zero-effect that is "closest to the middle" of the distribution of data). Similarly, if there were a series of successive complete mortalities at the high concentrations in the test, only one value of 100% effect would be used, i.e., the one at the lowest concentration. Use of only one 0% and one 100% effect applies to any form of statistical analysis and to hand plotting on a graph.

Various computer programs may be used to calculate the LC50. Stephan (1977) developed a program to estimate LC50s using probit, moving average, and binomial methods, and adapted it for the IBM-compatible personal computer. This program in the BASIC language is recommended, and is available on diskette[37] from Environment Canada (address in Appendix B). Other satisfactory computer and manual methods may be used (e.g., USEPA, 1985a; Hubert, 1987; APHA *et al.*, 1995; EC, 1997d). Programs using the trimmed Spearman-Kärber method (Hamilton *et al.*, 1977) are available for personal computers but are not recommended because divergent results might be obtained by operators who are unfamiliar with the implications of trimming ends of the dose-response data (EC, 1997d).

The recommended program of Stephan (1977) provides estimates of LC50 and confidence limits by each of its three methods if there are at least two partial mortalities in the set of data. For smooth or regular data, the three results will likely be similar, and values from the probit analysis should be taken as the preferred ones and reported. The probit analysis also gives the slope of the line, which should be reported. The binomial estimate might differ somewhat from the others, and this estimate should only be used as a last resort. If the results do not include two partial mortalities, only the binomial method can be used to provide an estimate of the LC50. Formal confidence limits are not estimated using the binomial method; instead, outer limits of a range are provided, within which the LC50 and the true confidence limits would lie.

Any computer-derived LC50 should be checked by examining a plot, on logarithmic-probability scales, of percent mortalities at Day 14 for the various test concentrations

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[37] Through the courtesy of Dr. Charles E. Stephan (USEPA, Duluth, MN).

(APHA *et al.*, 1995; EC, 1997d). Any major disparity between the estimated LC50 derived from this plot and the computer-derived LC50 must be resolved. A hand-plotted graph is preferred for this check. A computer-generated plot could be used if it were based on logarithmic-probability scales. If there had been an error in entering the data, however, a computer-generated plot would contain the same error as the mathematical analysis, and so the investigator should carefully check for correct placement of points.

A manual plot of mortality/concentration data to derive an estimated LC50 is illustrated in Figure 2. In this hypothetical example, there were 100 amphipods (five replicates of 20 organisms each, per concentration) tested at each of five concentrations. This figure was based on concentrations of 1.8, 3.2, 5.6, 10, and 18 mg chemical/kg sediment causing mortalities of 0, 20, 40, 90, and 100% of test amphipods exposed to the respective concentrations for 14 days. The concentration expected to be lethal to 50% of the amphipods can be read by following across from 50% (broken line) to the intersection with the best-fit line, then down to the horizontal axis for an estimated LC50 (5.6 mg/kg). A similar plot could be made of mortality/concentration data using the average measured concentration (in µg/L or mg/L) determined for the porewater analyses (see Section 6.4).

In fitting a line such as that in Figure 2, more emphasis should be assigned to points that are near 50% mortality. Logarithmic-probability paper (*log probit*, as in Figure 2) can be purchased in good technical bookstores, ordered through them, or photocopied (see blank graph in EC, 1997d).

For the regular set of data in Figure 2, computer programs gave very similar estimates to the graphic one. The LC50s (and 95% confidence limits) were:

Stephan (1977) method:

- probit: 5.58 (4.24 and 7.37)
- moving average: 5.58 (4.24 and 7.33)
- binomial: 6.22 (between 1.8 and 10)

Probit analysis
of Hubert (1987): 5.56 (4.28 and 7.21)

TOXSTAT 3.4: 5.58 (4.38 and 7.12)

SAS (1988)
probit analysis: 5.58 (4.26 and 7.40)

If a solvent control is included in the study, the dry weight of test organisms in replicates of the *solvent control* sediment should be compared statistically with weights from the *clean control* sediment. Student's *t*-test may be applied for this comparison (Section 5.6.1). If the weights for the two controls differ significantly, only the solvent control may be used as the basis for comparison and calculation of results. If the results are the same, the data from both controls should be used for assessing acceptability of the test and as the basis for calculating results (USEPA, 1994a). The test is rendered

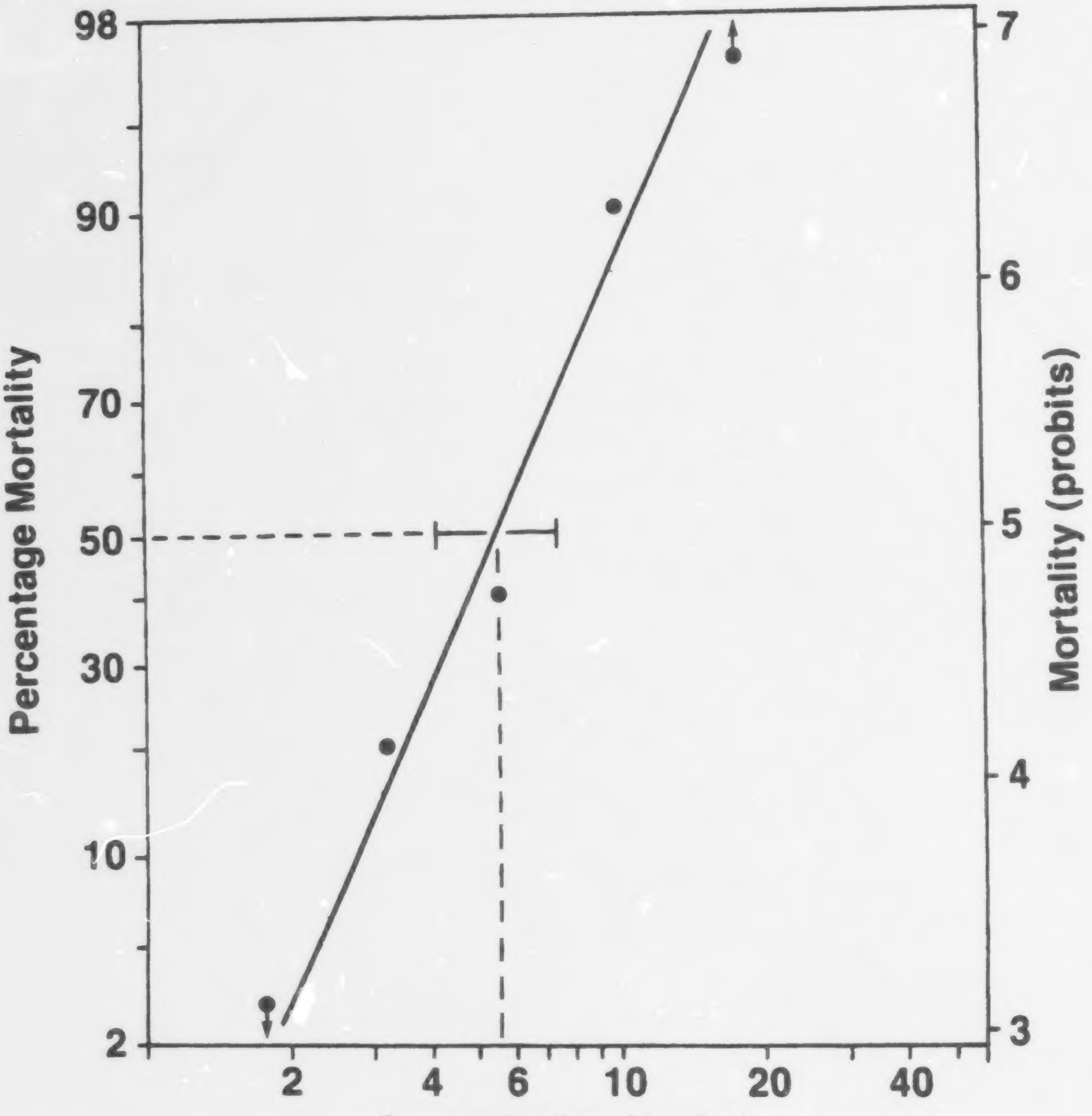

Figure 2 Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-probability Paper

invalid if more than 20% of the amphipods held in either control sediment die during the test.

6.5.2 Inhibiting Concentration for a Specified Percent Effect (ICp)

For the data on mean dry weight, the ICp (*inhibiting concentration for a specified percent effect*) is the recommended statistical endpoint. The ICp is a quantitative estimate of the concentration causing a fixed percent reduction in mean dry weight of test organisms (e.g., the IC25 and/or IC20,which represent 25% and 20% reduction). The desired value of p is selected by the investigator, and 25% or 20% is currently favoured. Any ICp that is calculated and reported must include the 95% confidence limits.

The mean weight of test organisms is calculated as the total dry weight of the amphipods that survived in a given chamber, divided by the number of organisms that survived to the end of the test (Section 4.7). If there are no survivors in a replicate (chamber), that replicate is excluded from the analysis. If there is complete mortality in all replicates at a given concentration, that concentration is excluded.

At present, the only easily available method of estimating the ICp and its 95% confidence limits is the "bootstrap" method on computer (Norberg-King, 1993), a program called *ICPIN* (USEPA, 1994b; c). ICPIN is not proprietary, is available from USEPA, and is included in most computer software for environmental toxicology, including TOXSTAT. The original instructions for ICPIN from USEPA are clearly written and make the program easy to use (Norberg-King, 1993).[38] An earlier version was called BOOTSTRP.

Analysis by ICPIN does not require equal numbers of replicates in different concentrations. The ICp is estimated by smoothing of the data as necessary, then using the two data-points adjacent to the selected ICp (USEPA, 1994b; c). The ICp cannot be calculated unless there are test concentrations both lower and higher than the ICp; both those concentrations should have an effect reasonably close to the selected value of p, preferably within 20% of it. At present, the computer program does not use a logarithmic scale of concentration, and so Canadian users must enter the concentrations as logarithms. Some commercial computer packages have the logarithmic transformation as a general option, but investigators should make sure that it is actually retained when proceeding to ICPIN. ICPIN estimates confidence limits by a special "bootstrap" technique because usual methods would not be valid.

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[38] The instructions in Norberg-King (1993) are sometimes misleading on the identity of "replicates". The term is used in such a way that it would apply to weights of individual organisms within the same chamber. This slip of wording does not affect the functioning of the program.

Commercial packages available in 1996 were more difficult for entry of data and analysis, than the original program of USEPA (Norberg-King, 1993). Some packages were recalcitrant, with manuals that offered minimal guidance. Current commercial programs generally require and produce particular information specified by USEPA which would not necessarily satisfy requirements of Environment Canada. A commercial program might also be written for reasonably modern personal computers (1996+); one required enough conventional memory (at least 610 free kilobytes) that little might be left for other programs.

Bootstrapping performs many resamplings from the original measurements. The investigator must specify the number of resamplings which can range from 80 to 1000. At least 400 is recommended and 1000 would be beneficial.[39]

Besides determining and reporting a computer-derived ICp, a graph of percent reduction of dry weight against the logarithm of concentration should be plotted, to check the mathematical estimation and to provide a visual assessment of the nature of the data (EC, 1997d).

If a *solvent control* is included in the study, the weight of surviving amphipods in its replicates should be compared statistically with weights from the control using clean sediment. *Student's t-test* may be applied for this comparison. If the weights differ significantly in the two controls, only the solvent control may be used as the basis for comparing and calculating results. If the results are the same, the data from both controls should be used for assessing the acceptability of the test and as the basis for calculating results (USEPA, 1994a). The test is rendered invalid if more than 20% of the amphipods held in either control sediment die during the test.

6.5.3 Hypothesis Testing (NOEC and LOEC)

An optional approach for presenting the results of the test is determining the *no-observed-effect concentration (NOEC), lowest-observed-effect concentration (LOEC), and threshold-observed-effect concentration (TOEC)*. For these spiked-sediment tests, NOECs and LOECs are calculated from the mean dry weights of surviving amphipods in each replicate (chamber) of the control and the various concentrations. Calculations use the same sublethal data used in estimating the ICp. If there is complete mortality in a replicate or a concentration, it is excluded from the analysis.[40]

Statistical procedures are explained with some guidance in USEPA (1994a; b; c), USEPA/USACE (1994), Newman (1995), EC (1997d), and in commercial software packages such as TOXSTAT (WEST, Inc. and Gulley, 1996). The methods start with a check of normality and homogeneity of variance by the *Shapiro-Wilks* and *Bartlett's tests*. If both tests are satisfied by the data in their original state or transformed (Section 5.6), analysis should proceed using parametric methods.

For parametric testing, an *analysis of variance (ANOVA)* is carried out, then *Williams' test*, a multiple-comparison test that determines which concentrations are significantly different from the control. Williams' test takes into account the order of concentrations by magnitude, a desirable feature to increase sensitivity, and a very appropriate attribute for most toxicity tests (Masters *et al.*, 1991).[41] Williams' test estimates the *Minimum Significant Difference (MSD)*. This is the magnitude of the difference in mean weights that would have to exist between the control and a test concentration, in order to conclude that there was a significant effect at that concentration. Any test which reports NOEC/LOEC must also report the MSD. If the MSD of average weights is >25% of the mean weight of the controls, the validity and usefulness of the findings is questionable.

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[39] ICPIN has some deficiencies. Its interpolation method is an inefficient use of data, sensitive to peculiarities of the two concentrations used. The program fails to adopt logarithm of concentration, which would introduce a slight bias towards a higher value of ICp. A modification of the bootstrap method has now remedied a problem of overly narrow confidence limits. Linear regression or general-purpose regression would be better methods of estimating the ICp and its 95% confidence limits (EC, 1997d), but a standard "packaged" method of regression has not been developed for environmental toxicology. Investigators should watch for any development of such a suitable new program.

[40] It is conceivable that significant mortality might occur at lower concentrations than those affecting mean weight. Although other methods of analysis might quantify such a effect on mortality, LC50 is the only endpoint for mortality that is to be reported for this sediment toxicity test.

If there are unequal numbers of replicates because of accidental loss or other causes, Williams' test is replaced by the *Dunn-Sidak modification of the t-test*, or by *Bonferroni's adjustment of the t-test*.

If tests for conformity and homogeneity cannot be satisfied by transformation, the parametric analysis should proceed except in cases of severe departure from normality which clearly would not fit such an analysis. The parametric tests are relatively robust in the face of moderate nonconformance (see Section 5.6). Nonparametric analysis should also proceed. The more sensitive (lower concentrations) of the two analyses is to be used as the final estimate of NOEC and LOEC. In this situation, detailed reporting must include the following items:

- results of the *Shapiro-Wilks* and *Bartlett's tests*;
- a hand-plotted graph of mean weights by chamber, using logarithmic concentration;
- findings of parametric analysis, including MSD; and
- findings of nonparametric analysis.

Nonparametric analysis requires four replicates.[42] *Shirley's test* would be the method of choice for use instead of analysis of variance. It parallels Williams' test in taking into consideration the ranking of concentration. Unfortunately, *Shirley's test* is not available in most statistical packages, nor is it described in most textbooks. *Steel's many-one rank test* is offered in most United States statistical packages, and could be used in this situation; it does not consider the order of concentrations. If there were unequal replication, the *Wilcoxon rank sum test* should be used. These tests are strong tools for data that are not normally distributed, but they would be less powerful than parametric tests if used on normally distributed data.

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[41] Another standard multiple-comparison test, *Dunnett's test*, is given more prominence in TOXSTAT and most methods from the United States. It is not a particularly powerful way of discriminating effects since it ignores the magnitudes of the concentrations when it calculates the MSD (Masters *et al.*, 1991).

[42] The requirement for four replicates might prevent an estimation of NOEC/LOEC. A test might have been designed with fewer replicates, primarily for calculating the ICp. If results were found to deviate from normality or homogeneity, the investigator would not be able to complete the analysis by nonparametric methods.

The geometric mean of the NOEC and LOEC, the *TOEC*, is often calculated for the convenience of having one number. The TOEC should be reported, recognizing that it is an arbitrary estimate of an effect-threshold that might lie anywhere in the range between the LOEC and NOEC. Its value is governed by whatever concentrations were selected for the test. No confidence limits can be estimated for the NOEC, LOEC, or TOEC.

Reporting Requirements

Each test-specific report must indicate if there has been any deviation from any of the *must* requirements delineated in Sections 2 to 6 of this biological test method, and, if so, provide details of the deviation. The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended.

Section 7.1 provides a list of items that must be included in each test-specific report. A list of items that must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years, is found in Section 7.2. Specific monitoring programs, related test protocols, or regulations might require selected test-specific items listed in Section 7.2 (e.g., details about the test material and/or explicit procedures and conditions during sample collection, handling, transport, and storage) to be included in the test-specific report, or might relegate certain test-specific information as *data to be held on file*.

Procedures and conditions common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes) and consistent with specifications in this document, may be referred to by citation or by attachment of a general report which outlines standard laboratory practice.

Details on the conduct and findings of the test, which are not conveyed by the test-specific report or general report, must be kept on file by the laboratory for a minimum of five years so that the appropriate information can be provided if an audit of the test is required. Filed information might include:

- a record of the chain-of-continuity for field-collected or other samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- chemical analytical data on the sample(s) not included in the test-specific report;
- bench sheets for the observations and measurements recorded during the test;
- bench sheets and warning chart(s) for the reference toxicity tests;
- detailed records of the source of the test organisms, their taxonomic confirmation, and all pertinent information on their culturing and health; and
- information on the calibration of equipment and instruments.

Original data sheets must be signed or initialled, and dated by the laboratory personnel conducting the tests.

7.1 Minimum Requirements for a Test-specific Report

Following is a list of items that must be included in each test-specific report.

7.1.1 Test Substance or Material

- brief description of sample type (e.g., dredged material, reference or contaminated field-collected sediment, control sediment) or coding, as provided to the laboratory personnel;
- information on labelling or coding of each sample; and
- date of sample collection; date and time sample(s) received at test facility.

7.1.2 Test Organisms

- species and source of brood stock and test organisms;
- range of age, at start of test;
- percentage of young amphipods in *known age* cultures that died or appear to be dead or inactive during the 48-h period immediately preceding the test; and
- any unusual appearance or treatment of the organisms, before their use in the test.

7.1.3 Test Facilities

- name and address of test laboratory; and
- name of person(s) performing the test.

7.1.4 Test Water

- type and source of test water; and
- measured characteristics of test water, before and/or at time of commencement of toxicity test.

7.1.5 Test Method

- citation of biological test method used (i.e., as per this document);
- design and description if specialized procedure (e.g., sieving of field-collected test sediment; preparation of mixtures of spiked sediment; preparation and use of solvent and, if so, solvent control) or modification of standard test method;
- brief description of frequency and type of observations and measurements made during test; and
- name and citation of program(s) and methods used for calculating statistical endpoints.

7.1.6 Test Conditions and Procedures

- design and description if any deviation from or exclusion of any of the procedures and conditions specified in this document;
- number of discrete samples per treatment; number of replicate test chambers for each treatment; number and description of treatments in each

test including the control(s); test concentrations (if applicable);

- depth and volume of sediment and overlying water in each test chamber;
- number of organisms per test chamber and treatment;
- test option chosen (i.e., daily-renewal or static);
- feeding regime and ration;
- indication of any aeration of overlying water (including rate);
- dates when test was started and ended;
- for each sample — all measurements of sediment particle size, percent water content, and total organic carbon; and of porewater pH and ammonia; and
- for at least one test chamber representing each treatment — all measurements of temperature and dissolved oxygen in overlying water, made at start of test and three or more times per week thereafter; all measurements of conductivity, pH, and ammonia in overlying water, made at start and end of test.

7.1.7 *Test Results*

- for each treatment — mean ± SD for percentage of amphipods that survived the 14-day exposure; mean ± SD for dry weight of surviving amphipods at test end; results of any statistical comparisons;
- coefficient of variation (CV) for mean percent survival and mean individual dry weight of replicate control groups at test end;
- any LC50 (including the associated 95% confidence limits and, if calculated, the slope) determined;
- any ICp (together with its 95% confidence limts) determined for the data on growth (i.e., dry weight at test end); details regarding any transformation of data that was required, and indication of quantitative statistic used;
- for a multi-concentration test with spiked sediment, indication as to whether results are based on nominal or measured concentrations of a particular substance or material;
- results for any 96-h LC50 (including its 95% confidence limits) performed with the reference toxicant(s) using the same batch of test organisms, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests using the procedures and conditions herein; and
- anything unusual about the test, any problems encountered, any remedial measures taken.

7.2 Additional Reporting Requirements

Following is a list of items that must be either included in the test-specific report or the general report, or held on file for a minimum of five years.

7.2.1 Test Substance or Material

- identification of person(s) who collected and/or provided the sample;
- records of sample chain-of-continuity and log-entry sheets; and
- conditions (e.g., temperature, in darkness, in sealed container) of sample upon receipt and during storage).

7.2.2 Test Organisms

- name of person(s) who identified the organisms and the taxonomic guidelines used to confirm species;
- history and age of brood stock;
- description of culture conditions and procedures for *mixed age* and *known age* cultures, including: facilities and apparatus, lighting, water source and quality, water pretreatment, water exchange rate and method, water temperature, age and density in culture, type and quantity of substrate;
- procedures used to count, handle, sort, transfer, and sieve animals; and those to determine their mortality, condition, appearance, and behaviour; and
- source and composition of food, procedures used to prepare and store food, feeding method(s), feeding frequency and ration.

7.2.2 Test Facilities and Apparatus

- description of laboratory's previous experience with this biological test method for measuring sediment toxicity using *H. azteca*;
- description of systems for providing lighting and compressed air, and for regulating temperature within test facility;
- description of test chambers, and covers if used;
- description of apparatus used to deliver and renew overlying water in test chambers, if the daily-renewal test option is chosen; and
- description of procedures used to clean or rinse test apparatus.

7.2.4 Control Sediment and Test Water

- procedures for pretreatment of control sediment (e.g., sieving, settling of sieved fines, formulation and aging if formulated) and test water (e.g., filtration, sterilization, reconstitution and aging if reconstituted, temperature adjustment, aeration rate and duration);

- type and quantity of any chemical(s) added to test water; and
- storage conditions and duration before use.

7.2.5 *Test Method*

- procedures used for mixing or otherwise manipulating test sediments before use; time interval between preparation and testing;
- procedure used in preparing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;
- methods used (with citations) for chemical analyses of test material (sediment and pore water) including details concerning aliquot sampling, preparation, and storage before analysis; and
- use and description of preliminary or range-finding test.

7.2.6 *Test Conditions and Procedures*

- measurements of light intensity adjacent to surface of overlying water in test chambers;
- statement concerning any aeration of overlying water in test chambers before and during the test; aeration rate and manner;
- records of any disruption of air flow to test chambers during static test, and of related DO measurements;
- description of procedure and rate for renewal of overlying water;
- appearance of each sample (or mixture thereof) and of the overlying water in test chambers; changes in appearance noted during test;
- any other chemical measurements (e.g., contaminant concentration, acid volatile sulphides, biochemical oxygen demand, chemical oxygen demand, total inorganic carbon, cation exchange capacity, redox potential, porewater hydrogen sulphide, porewater ammonia) made before and during the test on test material (including control and reference sediment) and contents of test chambers; including analyses of whole sediment, pore water, and overlying water;
- any other observations or analyses made on the test material (including samples of control or reference sediment); e.g., faunal tracks, qualitative and/or quantitative data regarding indigenous macrofauna or detritus, geochemical analyses; and
- chemical analyses of concentrations of chemical in test solutions of reference toxicant.

7.2.7 *Test Results*

- results for any range-finding test(s) conducted;
- warning chart showing the most recent and historic results for toxicity tests with the reference toxicant(s).

- graphical presentation of data; and
- original bench sheets and other data sheets, signed and dated by the laboratory personnel performing the test and related analyses.

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[43] A BASIC computer program for calculating LC50 is available from the Aquatic Toxicology Section, Pacific Environmental Science Centre, 2645 Dollarton Highway, North Vancouver, BC, V7H 1V2, by providing a formatted computer diskette.

Procedural Variations for Culturing *Hyalella azteca*, as described in Canadian and United States Methodology Documents

Source documents are listed chronologically by originating agency. They can be accessed as:

DFO 1989 represents Borgmann and Manawar (1989) and Borgmann *et al.* (1989). Together, these publications give the culturing and test procedures then in use by the Great Lakes Laboratory for Fisheries and Aquatic Sciences, Department of Fisheries and Oceans (DFO), Burlington, ON.

USFWS 1990 represents Ingersoll and Nelson (1990). This publication gives the culturing and test procedures then in use by the National Fisheries Contaminant Research Center, United States Fish and Wildlife Service, Columbia, MO.

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USEPA 1991a includes the draft (April 1991) standard operating procedures for culturing and testing *H. azteca* used by the USEPA Environmental Monitoring Systems Laboratory at Cincinnati, OH, See "USEPA 1991a" in list of references.

USEPA 1991b represents the draft (October 25, 1991) standard operating procedures used for culturing *H. azteca* by the United States Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN, See "USEPA 1991b" in list of references.

USEPA 1991c represents a summary presentation of the culturing procedures investigated by the USEPA Environmental Monitoring Systems Laboratory at Cincinnati, OH. See "Smith *et al.*, 1991a" in list of references.

DFO 1992 is the (unpublished) standard operating procedures used for culturing and testing *H. azteca* by U. Borgmann of the Great Lakes Laboratory for Fisheries and Aquatic Sciences, Department of Fisheries and Oceans (DFO), Burlington, ON. The procedure is the same as that published in Borgmann and Norwood (1993). See "DFO 1992" in list of references.

NWRI 1992 is the (unpublished) standard operating procedures used for culturing and testing *H. azteca* by K. Day of the National Water Research Institute (NWRI), Rivers Research Branch, Canada Centre for Inland Waters, Environment Canada, Burlington, ON. See "NWRI 1992" in list of references.

USEPA 1992 represents Norberg-King (1992), of the USEPA Environmental Research Laboratory at Duluth, MN. Procedures in use at 18 United States and Canadian laboratories for culturing and testing *H. azteca* are summarized.

USFWS 1992 represents Ingersoll (1992), of the United States Fish and Wildlife Service, Columbia, MO. Procedures in use at various laboratories for culturing and testing *H. azteca* are listed.

USEPA 1994a is the published methods for measuring the toxicity and bioaccumulation of sediment associated contaminants with freshwater invertebrates by the United States Environmental Protection Agency (principal authors, C.G. Ingersoll, G.T. Ankley, G.A. Burton, F.J. Dwyer, T.J. Norberg-King, and P.V. Winger). See "USEPA 1994a" in list of references.

1. Source of Brood Stock for Culture

Document[a]	Initial Source
DFO 1989	marshy shoreline of small lake near Burlington, Ontario
USFWS 1990	NI[b]
ASTM 1991	natural freshwater source, another laboratory, or a commercial source
USEPA 1991a	natural freshwater source, another laboratory, or a commercial source
USEPA 1991b	best source from a Lake Superior bay; acceptable sources, other laboratories, commercial suppliers, local collections
USEPA 1991c	USEPA Newtown strain
DFO 1992	marshy shoreline of small lake near Burlington, Ontario
NWRI 1992	CCIW Burlington laboratory (W. Norwood/U. Borgmann)
USEPA 1992	various (St. Louis River, 2 labs[c]; lake near Burlington, 2 labs; Michigan State pond, 1 lab; Nebeker strain, 8 labs; USEPA Newtown, 4 labs)
USFWS 1992	NI (various, depending on the laboratory)
USEPA 1994a	various (avoid wild populations unless the ability of the wild population to cross-breed with existing laboratory populations has been demonstrated

[a] See preceding page for correct citation.
[b] NI = Not indicated. Source was the USEPA Corvallis strain, as provided by A. Nebeker.
[c] labs = Laboratories.

2. Culture Vessels and Loading

Document	Vessel Type	Water Volume (L)	No. of Adult Amphipods/L
DFO 1989	2.5-L pyrex glass jar	1.0	5 to 25
USFWS 1990	80-L glass aquarium	50.	NI[a]
ASTM 1991	10-L or 20-L aquarium	NI	NI
USEPA 1991a	8-L aquarium	6 L	NI
USEPA 1991b	2-L battery jar or aquarium	1.0	60
USEPA 1991c	30-mL cup	0.02	100
	1-L glass beaker	NI	80
	8-L aquarium	6.0	17 to 33
	76-L aquarium	40.0	13 to 50
DFO 1992	2.5-L pyrex glass jar	1.0	5 to 25
NWRI 1992	10-L glass aquarium	8.0	20 to 25
	1.2-L glass jar	1.0	20 to 25
USEPA 1992	1-L to 39-L aquarium	0.8 to 38	NI
USFWS 1992	1-L to 100-L[b] aquarium	NI	NI
USEPA 1994a	2-L glass beaker	1.0	50
	2.5-L glass jar	1.0	5 to 25
	80-L aquarium	50.0	NI

[a] NI = Not indicated (depends on method used).

[b] Preferred choice.

3. Water Source, Hardness, and Method of Replacement During Culturing

Document	Water Source	Water Hardness	Method of Replacement
DFO 1989	dechl. tap[a]	130 mg/L	IR[d] (once weekly)
USFWS 1990	well	283 mg/L	FT[e] (~3 times/day)
ASTM 1991	well, surface, dechl. tap[b], or recon.[c]	optional	IR (25 to 30%/week), or FT (100 mL/min)
USEPA 1991a	as per ASTM 1991	optional	FT (100 mL/min), or IR (≥ 50%/week)
USEPA 1991b	surface or recon.	NI[f]	IR (once weekly)
USEPA 1991c	well or dilute well[g]	100 mg/L, 200 mg/L	IR (daily) or FT
DFO 1992	dechl. tap	130 mg/L	IR (once weekly)
NWRI 1992	dechl. tap	NI	IR (30%, once weekly)
USEPA 1992	dechl. tap (7), well (4), surface (3), recon. (3)	very soft to very hard	IR or FT
USFWS 1992	various	soft/hard[h]	IR or FT[h]
USEPA 1994a	well, surface, recon.[i], dechl. tap[b], estuarine[j]	optional	IR or FT[k]

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[a] Dechlorinated municipal tap water.

[b] Dechlorinated water should only be used as a last resort, since dechlorination is often incomplete.

[c] Reconstituted water.

[d] IR = Intermittent renewal.

[e] FT = Flow-through.

[f] NI = Not indicated.

[g] Well water with hardness 200 mg/L diluted to hardness 100 mg/L using deionized water.

[h] Preferred choice.

[i] A recipe is provided for preparing suitable reconstituted water with hardness 90 to 100 mg/L.

[j] *H. azteca* have been cultured in reconstituted salt water with salinities up to 15 ‰.

[k] Renewal of culture water, with at least one volume addition/d, is recommended. As a minimum, the overlying water volume should be changed at least weekly by siphoning.

4. Temperature, Aeration, and Lighting During Culturing

Document	Water Temp. (°C)	Aeration Conditions	Lighting
DFO 1989	25	none[a]	16L:8D[a], fluor.[b], 55 µE/m²/s[c]
USFWS 1990	20 ± 2	gentle (~2 bubbles/s)	16L:8D, 269 to 538 lux
ASTM 1991	20 ± 2	gentle, if IR[d]	16L:8D, 5382 lux
USEPA 1991a	25 ± 2	gentle, if IR	16L:8D, 5382 lux
USEPA 1991b	25	gentle (air stone)	16L:8D, 1280 lux
USEPA 1991c	25 (FT)[e] 23 (IR)	IR only	16L:8D, 538 to 1076 lux
DFO 1992	25	none	16L:8D, fluor., 55 µE/m²/s[c]
NWRI 1992	23 ± 1	gentle	16L:8D, 51 µE/m²/s
USEPA 1992	15 to 25[f]	NI[g]	NI
USFWS 1992	20[h] to 25	moderate	16L:8D, 538 to 1076 lux
USEPA 1994a	23	yes if static or IR	16L:8D, 500 to 1000 lux

[a] Daily photoperiod of 16 hours light and 8 hours dark.
[b] Overhead fluorescent tubes.
[c] In the laboratory used by these investigators, 1 µE/m²/s = 102.5 lux. Conversion could be different for different types of light.
[d] IR = Intermittent renewal.
[e] FT = Flow-through.
[f] One laboratory at 15°C, three at 20°C, one at 21 ± 2°C, eight at 23°C, four at 25°C.
[g] NI = Not indicated.
[h] Preferred choice.

5. Substrate for Amphipods During Culturing

Document	Description of Substrate Used	Size/Quantity of Substrate
DFO 1989	plastic and cotton gauze	several pieces in jar
USFWS 1990	hard maple leaves previously soaked for 30 d and rinsed for 1 h before use	NI[a]
ASTM 1991	dried maple, alder, birch or poplar leaves, pre-soaked several days and then rinsed	NI
USEPA 1991a	shredded brown paper towel	NI
USEPA 1991b	medicinal gauze sponges, 10 × 10 cm, pre-soaked in culture water for 24 to 48 h	1/jar
USEPA 1991c	single layer of unbleached brown paper towel	NI
DFO 1992	sterile 5 × 10 cm gauze bandage, or 5 × 10 cm piece of 210 µm Nitex™ nylon mesh	1/jar
NWRI 1992	2.5 × 2.5 cm strips of 500 µm Nitex™ nylon mesh, pre-soaked in culture water for 24 h	8/aquarium 1/jar
USEPA 1992	various (gauze, 4 labs[b]; leaves, 4 labs; paper towels, 2 labs; plastic mesh, 2 labs; Nitex™, 1 lab; Nitex™/sand/towels, 1 lab; sediment/towels, 1 lab; plastic/leaves, 1 lab; mesh/towel, 1 lab; none, 1 lab	NI
USFWS 1992	maple leaves[c], Nitex™ screen, cotton gauze, 3-M base web plastic	NI
USEPA 1994a	various (e.g., cotton gauze, maple leaves, artificial coiled-web material)	NI

[a] NI = Not indicated.
[b] Laboratories.
[c] Preferred choice.

6. Feeding During Culturing

Document	Description of Food Used	Quantity per Litre[a]	Feeding Frequency
DFO 1989	TetraMin™ fish food flakes[b]	20 mg	1 to 3×/week
USFWS 1990	hard maple leaves plus ground Tetra™ Standard Mix fish food	NI[c]	*ad libitum*
ASTM 1991	choice of dried maple, alder, birch or poplar leaves; rabbit pellets; ground cereal leaves; fish food pellets; brine shrimp; heat-killed *Daphnia*; green algae and spinach	NI	NI
USEPA 1991a	TetraMin™ fish food flakes + brine shrimp	3.3 mg	1×/day
USEPA 1991b	best success using filamentous algae and YCT[d]; cultured diatoms (*Synedra*) as alternative diet	10 mL YCT algal "pinch"	3×/week 1×/week
USEPA 1991c	ground fish food flakes plus dried algae (*Spirulina* sp.)	50 to 167 mg	2×/day
DFO 1992	TetraMin™ fish food flakes[b]	10 mg	1 to 3×/week
NWRI 1992	Nutrafin™ fish food flakes[b]	2 or 4 drops[e]	2×/week
USEPA 1992	various (single food type, 7 labs; multiple food types, 11 labs)[f]	varied	varied[g]
USFWS 1992	maple leaves[h], TetraMin™, rabbit chow, diatoms	NI	NI
USEPA 1994a	various (e.g., YCT plus algae; TetraMin™)	varied	varied

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[a] Amount of food added per litre of culture water.

[b] Flakes were ground and sifted through a 500 µm mesh nylon screen.

[c] NI = Not indicated.

[d] Yeast, Cerophyll™, and trout chow (USEPA diet for culturing *Ceriodaphnia dubia*).

[e] Two drops of a 100 mg Nutrafin™/mL slurry added per jar; 4 drops per aquarium.

[f] Food types include various rations of yeast, Cerophyll, algae, diatoms, wheat grass, diatom, alfalfa, TetraMin™, Nutrafin™, YCT, rabbit pellets, leaves, and paper towels.

[g] For intermittent-renewal cultures, feeding frequencies ranged from 1×/month to 2×/day (47% of labs fed 2×/week); for flow-through cultures, frequencies ranged from 1×/week to 1×/day.

[h] Preferred choice.

7. Harvesting Young for Tests

Document	Description of Procedure	Frequency	No. of Young per Litre[a]
DFO 1989	shake off substrate; filter through 275 µm mesh into petri dish; rinse and sort[b]	once/week	NI[c]
USFWS 1990	rinse portion of mixed-age culture off leaves; filter through 425 µm mesh to obtain animals ≤3 mm; hold overnight in 1-L beaker with aerated water	NI	NI
ASTM 1991	rinse portion of mixed-age culture off leaves; filter through sieves 250 µm (for juveniles) to 425 µm mesh (for adults); hold juveniles ≤24 h in beakers	NI	NI
USEPA 1991a	obtain from adults[d], or sieve daily	daily	NI
USEPA 1991b	pour contents of jar into shallow pan; gently rinse/shake animals off substrate; count and return adults to jar; count young and use or rear for 7 days more	once/week	NI
USEPA 1991c	sieve young released from paired adults	3×/week	33 to 120[e]
DFO 1992	as per Borgmann and Munawar (1989)	once/week	5 to 25
NWRI 1992	pour contents of jar onto 363 µm mesh screen and rinse animals from screen into petri dish; separate young by pipette; count adults; count young and hold	once/week	20 to 35
USEPA 1992	NI	NI	NI
USFWS 1992	NI	NI	NI
USEPA 1994a	various, to obtain 7- to 14-d amphipods	varied	varied

[a] Estimated number of young harvested per litre of culture water.

[b] Separated young kept in jars with 1 L water, 1 piece of 5×10 cm guaze and 20 mg TetraMin™ for 2 days before being used in bioassays, to ensure survival and determine numbers of available young.

[c] NI = Not indicated.

[d] Paired adults are placed in 1-L beakers, 25 pairs/beaker, and fed. After 24 h, collect released young.

[e] A 1-L beaker with daily replacement of food and water can yield 120 young/day.

Appendix D

Procedural Variations for Sediment Toxicity Tests using *Hyalella azteca*, as Described in Canadian and United States Methodology Documents

Source documents are listed chronologically by originating agency. They can be accessed as:

DFO 1989 represents Borgmann and Manawar (1989) and Borgmann *et al.* (1989). Together, these publications give the culturing and test procedures then in use by the Great Lakes Laboratory for Fisheries and Aquatic Sciences, Department of Fisheries and Oceans (DFO), Burlington, ON.

USFWS 1990 represents Ingersoll and Nelson (1990). This publication gives the culturing and test procedures then in use by the National Fisheries Contaminant Research Center, United States Fish and Wildlife Service, Columbia, MO.

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USEPA 1991b represents a summary presentation of the testing procedures in use at the USEPA Environmental Monitoring Systems Laboratory at Cincinnati, OH. See "Smith *et al.* 1991b" in list of references.

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NWRI 1992 is the (unpublished) standard operating procedures used for culturing and testing *H. azteca* by K. Day of the National Water Research Institute (NWRI), Rivers Research Branch, Canada Centre for Inland Waters, Environment Canada, Burlington, ON. See "NWRI 1992" in list of references.

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USEPA 1994a is the published methods for measuring the toxicity and bioaccumulation of sediment associated contaminants with freshwater invertebrates by the United States Environmental Protection Agency (principal authors, C.G. Ingersoll, G.T. Ankley, G.A. Burton, F.J. Dwyer, T.J. Norberg-King, and P.V. Winger). See "USEPA 1994a" in list of references.

1. Test Type, Age/Size of Test Animals, Vessel Type, Number of Amphipods per Vessel, Number of Replicates per Treatment, and Test Duration

Document[a]	Test Type	Age/Size of Animal	Test Vessel	No. per Vessel	No. of Replic.	Test Duration (days)
DFO 1989	static[b]	0 to 7 d	2.5-L jar	20	Ni[c]	28
USFWS 1990	static	≤3 mm[e]	1-L bkr[g]	20	4	29
	FT[d]	≤3 mm	1-L bkr	20	4	29
ASTM 1991	static	2 to 3 mm[f]	1-L bkr	20	4	≤10 to 30
	static	2 to 3 mm	20-L aquar.	100	≥2	≤10 to 30
	FT	2 to 3 mm	1-L bkr	20	4	≤10 to 30
USEPA 1991a	IR[h]	2 ± 1 d	600 mL	20	4	7
USEPA 1991b	IR	2 ± 1 d	600 mL	20	4	7
DFO 1992	static	0 to 7 d	250-mL bkr	20	4	28
NWRI 1992	static[b]	1 to 10 d	250-mL bkr	15	5	28
USEPA 1992	static[i]	variable[j]	NI	NI	NI	10 to 28 [k]
USFWS 1992	FT[l]	7 to 14 d[m]	1 L[n]	20	4 to 5	7 to 28 [o]
USEPA 1994a	IR or FT[p]	7 to 14 d	300 mL	10	8[q]	10

[a] See preceding page for correct citation.

[b] Distilled water was added as needed to keep the water level constant.

[c] NI = Not indicated.

[d] FT = Flow-through.

[e] About third instar.

[f] Juvenile animals, second or third instar.

[g] Bkr = Beaker.

[h] IR = Intermittent renewal.

[i] Ten of 12 laboratories did not replace any water, two topped off. Nine of 18 labs also performed tests where water was renewed at frequencies ranging from every 4-6 h to twice per week.

[j] Seven labs, known age; 8 labs, sieve for size/age; 2 labs, mixed age; 1 lab, unknown.

[k] Eight labs, 10 d; 1 lab, 10 to 14 d; 4 labs, 14 d; 1 lab, 20 d; 4 labs, 28 d.

[l] Also static or static-renewal.

[m] Preferred choice, mixed age (~7 to 14 d); also known age (0 to 7 d or 7 to 14 d).

[n] Preferred choice; can range from 25 mL to 100 L.

[o] Preferred choice, 10 days.

[p] Two volume additions/d required, by intermittent (IR) or continuous (FT) replacement.

[q] Depends on test objective. Eight replicates are recommended for routine testing.

2. Test Vessels and Materials

Document	Vessel	Cover	Amount of Sediment	Amount of Water
DFO 1989	2.5-L pyrex screwtop jar	plexiglass sheet	1 to1.5 cm layer	NI[a,b]
USFWS 1990	1-L glass beaker	watch glass	200 mL	800 mL
ASTM 1991	1-L glass beaker 20-L aquarium	watch glass NI	200 mL 2 to 3 cm layer	800 mL 15-cm layer
USEPA 1991a	600 mL	watch glass or glass/plastic sheet	100 mL	400 mL
USEPA 1991b	600 mL	NI	100 mL	400 mL
DFO 1992	250-mL beaker	plastic petri dish[c]	40 mL[d]	160 mL[d]
NWRI 1992	250-mL beaker	petri dish[e]	50 mL	200 mL
USEPA 1992	NI	NI	NI	NI
USFWS 1992	1 L[f]	NI	NI[g]	NI[g]
USEPA 1994a	300-mL high-form lipless beaker	NI	100 mL	175 mL

[a] NI = Not indicated.
[b] Total volume (sediment plus seawater), 1.5 L.
[c] Notch cut out for air supply.
[d] Water added to beaker, then sediment introduced.
[e] Hole drilled for passage of airline tubing.
[f] Preferred choice; can range from 25 mL to 100 L.
[g] Water:sediment ratio can range from 4:1 (preferred choice) to 1:1.

3. Water Source, Hardness, and Method of Replacement During Test

Document	Water Source	Water Hardness	Method of Replacement
DFO 1989	dechlorinated tap[a]	130 mg/L	static with top up[b]
USFWS 1990	reconstituted[c]	134 mg/L	static, FT[d,e]
ASTM 1991	well, surface, dechlorinated tap, or reconstituted	optional	static or flow-through
USEPA 1991a	diluted well[f]	90 to 110 mg/L	IR[g]
USEPA 1991b	diluted well[f]	100 mg/L	IR, daily
DFO 1992	dechlorinated tap[a]	130 mg/L	static[h]
NWRI 1992	dechlorinated tap[a]	NI[i]	static[j]
USEPA 1992	NI	NI	static or renewal[k]
USFWS 1992	NI	soft, hard[l]	static, IR, FT[l]
USEPA 1994a	culture, well, surface, site, or reconstituted[m]	optional	IR or FT, 2×/d[n]

[a] Same source and hardness as used for culturing amphipods.

[b] Distilled water was added as needed to keep water level constant.

[c] Well water with hardness 283 mg/L was used for culturing amphipods; reconstituted water was used during the test.

[d] FT = Flow-through.

[e] 3.8 volume additions per beaker, per day.

[f] Well water with hardness 200 mg/L diluted to hardness 100 mg/L using deionized water.

[g] IR = Intermittent renewal.

[h] Water in controls only should be replaced weekly.

[i] NI = Not indicated.

[j] Water lost by evaporation was replaced weekly using distilled water.

[k] Of 18 laboratories surveyed, 10 used static with no replacement and 2 used static with top up. Nine of the 18 laboratories also renewed overlying water at a frequency ranging from every 4 to 6 h to 2 times/week.

[l] Preferred choice.

[m] A recipe was provided for preparing suitable reconstituted water with hardness 90 to 100 mg $CaCO_3$/L.

[n] Each test chamber should receive 2 volume additions/d of overlying water, using an intermittent-renewal (manual or automated) or continuous-flow system for replacements.

4. Temperature, Aeration, and Lighting During Test

Document	Water Temp. (°C)	Aeration Conditions	Lighting
DFO 1989	21 ± 1	gentle, using aquarium airstone suspended several cm above sediment	16L:8D[a], fluor.[b], 55 μE/m²/s
USFWS 1990	20 ± 2	gentle (~2 bubbles/s)	16L:8D, 269 to 538 lux
ASTM 1991	20 to 25	gentle	16L:8D, 538 lux
USEPA 1991a	25 ± 1	gentle	16L:8D, 538 lux
USEPA 1991b	25 ± 1	none	NI
DFO 1992	25	gentle, using disposable glass pipette with tip at midpoint of water column	16L:8D, fluor.
NWRI 1992	23 ± 1	gentle	16L:8D
USEPA 1992	20 to 25[c]	NI[d]	NI
USFWS 1992	20 to 25[e]	none or moderate[f]	16L:8D, 269 to 538 lux
USEPA 1994a	23 ± 1[g]	normally, none[h]	16L:8D, ~500 to 1000 lux, wide-spectrum fluorescent

---

[a] Daily photoperiod of 16 hours light and 8 hours dark.

[b] Overhead fluorescent tubes.

[c] Seven laboratories at 20°C, one at 20 to 25°C, four at 23°C, five at 25°C.

[d] NI = Not indicated.

[e] Preferred choice, 20°C.

[f] Preferred choice, none.

[g] Daily mean temperature must be 23 ± 1°C; instantaneous temperature must always be 23 ± 3°C.

[h] Aerate if dissolved oxygen in overlying water drops below 40% of saturation.

5. Feeding During Test

Document	Description of Food Used	Quantity per Vessel	Feeding Frequency
DFO 1989	TetraMin™ fish food flakes[a]	20 mg	1 to 3×/week
USFWS 1990	Purina™ rabbit pellets	14 or 20 mg[b]	3×/week
ASTM 1991	rabbit pellets[c]	varied[b,d]	2 to 3×/week
USEPA 1991a	ground TetraMin™ fish food flakes	14 mg	3×/week
USEPA 1991b	blended fish food flakes	1 mL	Days 0, 2, 4, and 6
DFO 1992	TetraMin™ fish food flakes[a]	5 mg	3×/week
NWRI 1992	Nutrafin™ fish food flakes[a]	8 mg[e]	2×/week
USEPA 1992	NI[f]	NI	varied[g]
USFWS 1992	varied[h]	NI	NI
USEPA 1994a	YCT[i]	1.5 mL	daily

[a] Flakes were ground and sifted through a 500 µm mesh nylon screen.

[b] 14 mg/beaker for static tests; 20 mg/beaker for flow-through tests.

[c] Pellets should be ground, dispersed in deionized water, and resuspended when aliquots are taken.

[d] Options include 6 mg pellets 3×/week for first week, and 12 mg per feeding thereafter.

[e] Added as a slurry of ground Nutrafin™, prepared by adding 1 g flakes to 100 mL distilled water and pulverizing. A volume of ~604 µL is equivalent to 8 mg.

[f] NI = Not indicated.

[g] Of 16 laboratories surveyed, five fed 7×/week during tests, five fed 3×/week, two fed 2×/week, one fed 1×/week, one fed every 48 h, one fed at start only, and one did not feed during test.

[h] None; rabbit chow; yeast, Cerophyll™, and trout chow (YCT); maple leaves; or TetraMin™.

[i] Yeast, Cerophyll™, and trout chow.

6. Monitoring Quality of Overlying Water During Test

Document	Variables Monitored[a]	Frequency
DFO 1989	NI[b]	NI
USFWS 1990	DO pH alk hard cond	at least every 10 days, each treatment[c]
ASTM 1991	DO pH alk hard cond temp	beginning, end, and at least weekly[d,e] beginning, end, and at least weekly[d] daily[d,f]
USEPA 1991a	DO pH alk hard cond temp	beginning and end
USEPA 1991b	NI	NI
DFO 1992	DO pH ammonia	beginning and at least weekly[c] beginning (optional but desirable)
NWRI 1992	DO pH cond temp	Days 0, 14, and 28
USEPA 1992	NI	NI
USFWS 1992	NI	NI
USEPA 1994a	DO[g] pH[g] alk hard cond ammonia temp	daily beginning and end[h] daily[d,f]

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[a] DO = dissolved oxygen; pH = hydrogen ion concentration; alk = total alkalinity; hard = total hardness; cond = specific conductivity; temp = temperature.

[b] NI = Not indicated.

[c] 50-mL volume of overlying water removed for measurements. In static exposure, this was replaced with fresh, temperature-adjusted overlying water.

[d] Measured in at least one test vessel representing each treatment.

[e] DO to be measured if any interruption of air (static test) or water (flow-through test) and whenever behaviour of animals indicates DO too low (e.g., if amphipods are seen to have emerged from sediment).

[f] Daily mean temperature must be within ±1 °C of desired temperature; instantanteous temperature must be within ±3 °C of desired temperature.

[g] Can be measured directly, using a probe.

[h] Overlying water should be sampled just before water renewal from about 1 to 2 cm above sediment surface, using a pipet. Values should not vary by more than 50% during a test.

7. Storage and Characterization of Sediment Used in Test

Document	Storage Conditions	Characteristics Measured[a]
DFO 1989	fridge, plastic bags	NI[b]
USFWS 1990	4°C in Teflon™ bags by 24 h; use within 2 weeks	TOC IC W SSC M PCB PAH
ASTM 1991	4 ± 2°C for ≤2 weeks[c]	at least pH TOC W SSC; might include BOD COD IC TVS AVS Eh OG OS TA M PAH PW
USEPA 1991a	4°C for ≤2 weeks	NI
USEPA 1991b	NI	NI
DFO 1992	NI	W TVS SG
NWRI 1992	sealed plastic buckets, 4°C; use within 6 weeks	NI
USEPA 1992	NI	NI
USFWS 1992	NI	NI
USEPA 1994a	4°C[d]	at least porewater pH + TA, and TOC W SSC; might include BOD COD CEC IC TVS AVS Eh OG SOC M PAH PW

[a] TOC = total organic carbon; IC = inorganic carbon; W = % water; SSC = % sand, silt, and clay; pH = hydrogen-ion concentration; BOD = biochemical oxygen demand; COD = chemical oxygen demand; CEC = cation exchange capacity; TVS = total volatile solids; SG = specific gravity (g/mL); AVS = acid volatile sulphides; Eh = oxidation reduction potential; OG = oil and grease; OS = organosilicones; TA = total ammonia; M = metals (e.g., As, Cd, Cr, Cu, Hg, Ni, Pb, Zn); PCB = total polychlorinated biphenyls; PAH = polycyclic aromatic hydrocarbons; SOC = synthetic organic compounds; PW = porewater analyses.

[b] NI = Not indicated.

[c] If stored longer than 2 weeks, sediment should be retested to confirm that toxicity has not changed.

[d] Start test as soon as possible following sample collection. If toxicity test is started after 2 weeks of collection, it is desirable to conduct additional characterizations of sediment to evaluate possible effects of storage.

8. Manipulation of Sediment Before Use in Test

Document	Sediment Manipulation
DFO 1989	wet-sieved through 275 μm nylon screen, using overlying water in test jars; allowed to settle several days before adding amphipods
USFWS 1990	mixed in storage container; aliquot smoothed with Teflon™ spoon in test beaker; overlying water poured gently along side of beaker; allowed to settle overnight before adding amphipods
ASTM 1991	mix thoroughly; may be wet-press sieved to remove large particles and indigenous organisms; may be diluted and mixed in a 1:1 ratio with overlying water to facilitate sieving; smooth as layer in test vessel; pour overlying water gently along side of beaker; allow to settle overnight before adding amphipods
USEPA 1991a	mix sample; smooth aliquot as layer in test vessel; pour overlying water gently along side of beaker; allow to settle overnight before adding amphipods
USEPA 1991b	NI
DFO 1992	mix sample or take aliquots from several locations; using a stainless steel spoon, transfer aliquot to beaker already containing overlying water; aerate overlying water vigorously for 24 h; reduce aeration to gentle flow and allow sediment to settle for further 24 h
NWRI 1992	wet-sieve through 250 μm mesh using portion of overlying water for test; discard residue retained and leave sieved sediment and overlying water undisturbed overnight; decant overlying water for use in test; add sediment to replicate beakers and then overlying water; allow to settle 24 h and aerate minimum of 1 h before adding amphipods[a]
USEPA 1992	NI[b]
USFWS 1992	NI
USEPA 1994a	mix, including any separated water; samples should not be sieved[c]; remove large organisms and large debris using forceps

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[a] In some situations, the sieved sediment is frozen for 24 h and thawed to kill all residual eggs of tubificids.

[b] NI = Not indicated.

[c] If sediment must be sieved, samples should be taken before and after sieving (e.g., pore-water metals) to document the influence of sieving on sediment chemistry.

9. Terminating Test and Biological Endpoints

Document	Terminating Test	Biological Endpoints
DFO 1989	sieve contents of jar through 275 µm mesh screen; sort, count, and weigh survivors	mean percent survival mean wet weight
USFWS 1990	wet-sieve sediment; preserve animals in sugar-formalin for subsequent measurement of length[a]	percent survival body length (mm)
ASTM 1991	pipet surviving animals from water column; sieve sediment using 500 µm mesh screen; count live and dead animals; measure length[b]	mean percent survival mean body length mean weight maturation
UEPA 1991a	sieve contents of beaker through 500 µm mesh screen; rinse animals from screen; count live and dead animals; measure mean dry weight[b]	mean percent survival mean dry weight
USEPA 1991b	NI	mean percent survival mean dry weight
DFO 1992	sieve contents of jar through 275 µm mesh screen; sort, count, and weigh survivors	mean percent survival mean wet weight
NWRI 1992	sieve contents of beaker through 500 µm mesh screen; count and weigh surviving amphipods	mean percent survival mean dry weight[c]
USEPA 1992	NI[d]	NI
USFWS 1992	NI	percent survival length or weight maturation
UEPA 1994a	pipet amphipods from water or sediment surface; sieve sediment through 710 µm mesh screen or using multiple sieves; count survivors and measure growth[e]	mean percent survival length or weight[e]

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[a] Animals not recovered are presumed to have died and decomposed.

[b] Additional screen sizes may be used for sieving. Animals may be preserved for subsequent determinations of length or weight.

[c] Surviving animals from each beaker are dried for 24 h at 60°C, then weighed as a group.

[d] NI = Not indicated.

[e] Survivors can be preserved in 8% sugar formalin solution for growth (i.e., body length) measurements. If determining dry weight, pool survivors and dry at 60 to 90°C to constant weight, bring to room temperature and weigh to nearest 0.01 mg. Measurement of growth is optional.

10. Use of Control/Reference Sediment and Requirement for Valid Test

Document	Control/Reference Sediment	Requirement for Valid Test
DFO 1989	none used[a]	NI[b]
USFWS 1990	fine-grained control sediment used	NI
ASTM 1991	every test requires a negative (clean) control sediment or a clean reference sediment[c]	mean survival ≥ 80%[d]
USEPA 1991a	every test requires a negative (clean) control sediment or a clean reference sediment[c]	mean survival ≥ 80%[d]; single-vessel survival, >70%[d]
USEPA 1991b	negative control sediment used	NI
DFO 1992	none used[a,e]	NI
NWRI 1992	negative control and reference sediments used	mean survival ≥ 80%[d]
USEPA 1992	negative control sediment used	mean survival 60 to 90%[f]
USFWS 1992	negative control sediment used	mean survival ≥ 80%[d]
USEPA 1994a	negative control and reference sediments used	mean survival ≥ 80%[d]

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[a] Control survival and growth was measured using gauze as substrate for animals (no sediment).

[b] NI = Not indicated.

[c] A reference sediment should be collected from the field in a clean area, and represent the test sediment in sediment characteristics (e.g., particle size, total organic carbon, pH).

[d] The test is unacceptable if the average survival of organisms in any test vessel containing negative control sediment is less than 80%.

[e] Water in controls (but not the beakers with sediment) was replaced weekly.

[f] Thirteen laboratories used 80% control survival for valid test, two used 70%, one used 90%, and one used 60%.

Procedural Variations for Reference Toxicity Tests with *Hyalella azteca*, as described in Canadian and United States Methodology Documents

Source documents are listed chronologically by originating agency. They can be accessed as:

ASTM 1991 is the (then) standard guide published by the American Society for Testing and Materials (Philadelphia, PA) for conducting sediment toxicity tests with freshwater invertebrates. This guideline document was published again in 1993. See "ASTM 1991a" and "ASTM 1993" in list of references.

USEPA 1991a includes the draft (April 1991) standard operating procedures for culturing and testing *H. azteca* used by the USEPA Environmental Monitoring Systems Laboratory at Cincinnati, OH. See "USEPA 1991a" in list of references.

USEPA 1991b represents a summary presentation of the testing procedures in use at the USEPA Environmental Monitoring Systems Laboratory at Cincinnati, OH. See "Smith *et al.* 1991b" in list of references.

DFO 1992 is the (unpublished) standard operating procedures used for culturing and testing *H. azteca* by U. Borgmann of the Great Lakes Laboratory for Fisheries and Aquatic Sciences, Department of Fisheries and Oceans (DFO), Burlington, ON. The procedure is the same as that published in Borgmann and Norwood (1993). See "DFO 1992" in list of references.

NWRI 1992 is the (unpublished) standard operating procedures used for culturing and testing *H. azteca* by K. Day of the National Water Research Institute (NWRI), Rivers Research Branch, Canada Centre for Inland Waters, Environment Canada, Burlington, ON. See "NWRI 1992" in list of references.

USEPA 1992a represents Norberg-King (1992), of the USEPA Environmental Research Laboratory at Duluth, Minn. Procedures in use at 18 United States and Canadian laboratories for culturing and testing *H. azteca* are summarized.

USEPA 1992b represents Smith *et al.* (1992a), of the USEPA Environmental Monitoring Systems Laboratory at Cincinnati, OH.

USFWS 1992 represents Ingersoll (1992), of the United States Fish and Wildlife Service, Columbia, MO. Procedures in use at various laboratories for culturing and testing *H. azteca* are listed.

USEPA 1994a is the published methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates by the United States Environmental Protection Agency (principal authors, C.G. Ingersoll, G.T. Ankley, G.A. Burton, F.J. Dwyer, T.J. Norberg-King, and P.V. Winger). See "USEPA 1994a" in list of references.

1. Reference Toxicant(s), Test Type, Duration, and Frequency of Use

Document[a]	Reference Toxicant(s)	Test Type	Test Duration	Frequency of Use
ASTM 1991	none	none	--	--
USEPA 1991a	$CuSO_4$, KCl, NaCl, Na dodecyl sulphate	water only[b]	96 h	≥ 1×/month[c]
USEPA 1991b	NI	IR[d], water only	96 h	1×/week
DFO 1992	NI[e]	NI	NI	NI
NWRI 1992	$CuSO_4$	static, water only	48 h	monthly[f]
USEPA 1992a	variable[g]	NI	NI	NI
USEPA 1992b	KCl	IR, spiked soil[h]	7 days	NI
USFWS 1992	NI	NI	NI	NI
USEPA 1994a	NaCl, KCl, Cd, Cu[i]	static, water only	96 h	monthly[j]

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[a] See preceding page for correct citation.

[b] Amphipods are exposed to a range of concentrations of the reference toxicant dissolved in fresh water; no sediment is present in the test.

[c] If preferred, this test may be performed concurrently with the sediment toxicity tests.

[d] IR = Intermittent renewal, with daily replacement of each test solution.

[e] NI = Not indicated.

[f] Chronic reference toxicant tests with sediment are performed biannually, using $CuSO_4$ and $CdCl_2$.

[g] Of 18 laboratories surveyed, six used Cd, one used Cr, three used Cu, five used KCl, one used NaCl, and one used Zn. Presumably, the remaining four laboratories did not use a reference toxicant.

[h] A dry, artificial soil was spiked with serial concentrations of the reference toxicant in solution. Each concentration, which represented the overlying water in the beaker, was replaced daily during the test.

[i] Sodium chloride, potassium chloride, cadmium chloride, and copper sulphate are suitable for use. It might be unrealistic to test more than one or two reference toxicants routinely (i.e., monthly).

[j] Ideally, tests with reference toxicants should be conducted in conjunction with sediment tests.

2. Age/Size of Test Animals, Vessel Type, Volume of Test Material(s), Number of Amphipods per Vessel, and Number of Replicates per Treatment

Document	Age/Size of Animal	Test Vessel	Volume of Solution	Volume of Sediment	No. Per Vessel	No. of Replicates
USEPA 1991a	NI[a]	NI	NI	none	NI	NI
USEPA 1991b	2 ± 1 d	NI	20 mL	none	5	4
NWRI 1992	1 to 10 d	250-mL beaker	200 mL	none	15	3 to 5
USEPA 1992b	3 to 7 d[b]	175-mL beaker	100 mL	25 mL	20	4
USEPA 1994a	7 to 14 d	250-mL beaker[c]	≥ 100 mL[c]	none	≥ 10[c]	≥ 3[c]

[a] NI = Not indicated.

[b] Seven-day old animals increase the power of the test, due to decreased variability.

[c] Tests can also be conducted using 30-mL plastic cups, 20 mL/cup, 1 amphipod/cup, and ≥ 10 replicates/cup.

3. Source and Hardness of Water Used in Test, and Variables Monitored

Document	Water Source	Water Hardness	Variables Monitored[a]	Monitoring Frequency
USEPA 1991a	NI[b]	NI	NI	NI
USEPA 1991b	diluted well[c]	100 mg/L	NI	NI
NWRI 1992	dechlorinated tap[d]	NI	DO pH cond	beginning and end
USEPA 1992b	diluted well[e]	100 mg/L	NI	NI
USEPA 1994a	culture, well, site, surface, reconst.[f]	optional[f]	pH alk hard cond temp DO	beginning and end daily

[a] DO = dissolved oxygen; pH = hydrogen ion concentration; cond = specific conductivity; alk = total alkalinity; hard = total hardness; temp = temperature.

[b] NI = Not indicated.

[c] Mixture of well water and deionized water.

[d] Same source and hardness as used for culturing amphipods.

[e] Mixture of well, dechlorinated tap, and deionized water.

[f] A recipe is provided for preparing suitable reconstituted water with hardness 90 to 100 mg $CaCO_3$/L.

4. Temperature, Aeration, and Lighting During Test with Reference Toxicant

Document	Water Temp. (°C)	Aeration Conditions	Lighting
USEPA 1991a	NI[a]	NI	NI
USEPA 1991b	25 ± 1	NI	NI
NWRI 1992	NI	NI	NI
USEPA 1992b	25 ± 1	NI	16L:8D
USEPA 1994a	23	none	16L:8D, ~500 to 1000 lux, wide-spectrum fluorescent

[a] NI = Not indicated.

5. Substrate Used in Test with Reference Toxicant

Document	Description of Substrate Used	Size/Quantity of Substrate
USEPA 1991a	NI[a]	NI
USEPA 1991b	NI	NI
NWRI 1992	2.5 × 2.5 cm strip of 500 µm Nitex™ nylon mesh, presoaked in culture water for 24 h	1/beaker
USEPA 1992b	dry, artificial soil, spiked with serial concentrations of the reference toxicant[b]	25 mL/replicate
USEPA 1994a	Nitex™ screen (110 mesh)	NI

[a] NI = Not indicated.
[b] Each concentration, which represented the overlying water and pore water, was replaced daily.

6. Feeding During Test with Reference Toxicant

Document	Description of Food Used	Quantity per Vessel	Feeding Frequency
USEPA 1991a	NI[a]	NI	NI
USEPA 1991b	*S. capricornutum* and cereal leave extract	0.1 mL algae 0.1 mL cereal	daily
NWRI 1992	NI	NI	NI
USEPA 1992b	algae and Cerophyll™	1 mL	daily
USEPA 1994a	YCT[b]	0.5 mL[c]	Days 0 and 2

[a] NI = Not indicated.
[b] Yeast, Cerophyll™, and trout chow; 1800 mg/L stock.
[c] For 250-mL beaker with 10 amphipods. Use 0.1 mL YCT if 30-mL cup with 1 amphipod.

7. Endpoints and Requirement for Valid Test Using Reference Toxicant

Document	Biological Endpoints	Statistical Endpoints	Requirement for Valid Test
USEPA 1991a	NI[a]	EC50[b]	NI
USEPA 1991b	survival	LC50[c]	NI
NWRI 1992	mean percent survival	EC50	mean survival ≥90%[d]
USEPA 1992b	survival[e]	IC50[f], IC25[f], NOEC[g]	NI
USEPA 1994a	survival	LC50	mean survival ≥90%[d]

[a] NI = Not indicated.
[b] Median effective concentration.
[c] Median lethal concentration.
[d] For controls used in test with reference toxicant.
[e] Mean dry weight was shown to be an insensitive endpoint in tests with KCl.
[f] Inhibiting concentration for a (specified) percent effect (i.e., that causing 50% or 25% inhibition).
[g] No-observed-effect concentration.

Appendix F

General Systematics of *Hyalella azteca* (Saussure, 1858)[44]

Taxonomy and Phyletic Relationships

Hyalella azteca (Saussure, 1858) is a member of the talitroidean amphipod family Hyalellidae (Bulycheva, 1957). Superfamily Talitroidea also includes the families Talitridae (beach fleas and sandhoppers), Dogielinotidae (North Pacific sand-burrowers), Najinidae (North Pacific algal borers), and the Hyalidae (kelp grazers). At the time of Bulycheva's revision, the family Hyalellidae contained about 20 described species, all in the genus *Hyalella* and all endemic to South American fresh waters (mostly in Lake Titicaca), except for the North American species, *H. azteca*. The family Hyalellidae was redefined by Bousfield (1979; 1982) to include also the Caribbean coastal marine genus *Parhyalella*, and the antipodean fresh- and brackish-water genera *Chiltonia* (New Zealand), *Austrochiltonia* (Australia), and *Afrochiltonia* (South Africa). These last three genera (of chiltoniins) had been placed in the inquilinous marine family Ceinidae by Barnard (1972) and Barnard and Barnard (1983). Bousfield (1996) has redefined the genus *Hyalella* which now includes about 35 described species (in four genera), nearly all confined to South America, but five of which (including *H. azteca*) occur in North American fresh waters. The genus is believed to have "split off" from the similar Pacific coastal marine genus *Allorchestes*, probably during the Lower Cretaceous (Gondwana times), when South America was beginning its isolation from the outer southern continental masses (Bousfield, 1984). *H. azteca* is almost certainly a later addition to North American fresh waters into which its immediate ancestors penetrated (northwards from South America) following closure of the Panama isthmus during Pliocene-Miocene epochs.

The basic body parts of *H. azteca* are illustrated in Figure F.1. This species has the typical talitroidean (vs. gammaroidean) features of:

(1) short antenna 1 that lacks an accessory flagellum;

(2) mouthparts in which the mandible has a strong molar but lacks a palp, and the palp of maxilla 1 is vestigial;

(3) gnathopods 1 and 2 that are regularly subchelate and subsimilar in females and immatures, but very unequal in mature males (gnathopod 2 much the larger and more powerful);

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[44] Prepared by E.L. Bousfield, Research Associate, Royal British Columbia Museum, 675 Bellevue Street, Victoria, BC V8V 1X4.

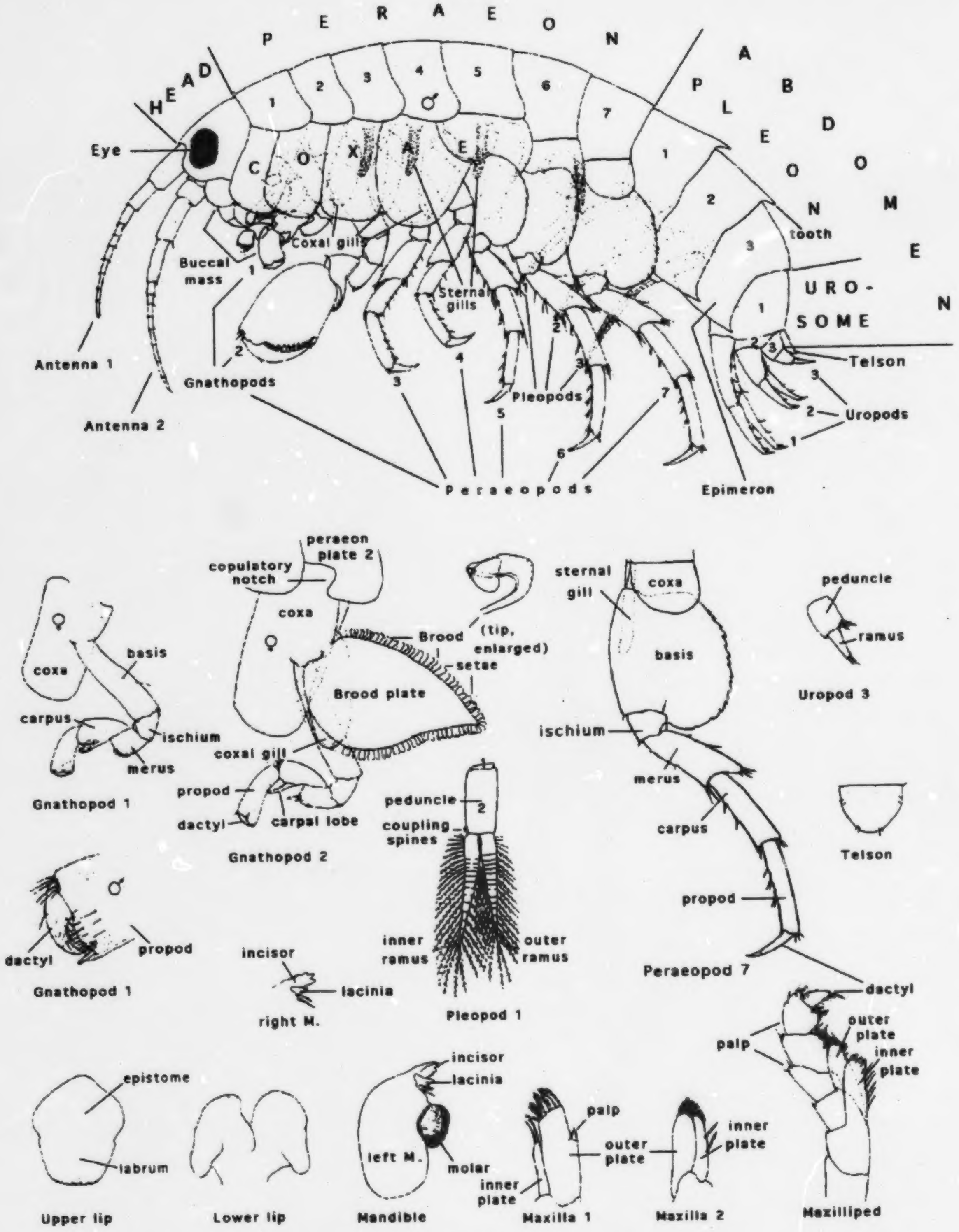
PERAEON
HEAD
1
2
3
4
5
6
7
♂
COXAE
Eye
Buccal mass
Coxal gills
Sternal gills
Antenna 1
Antenna 2
Gnathopods
1
2
Peraeopods
3
4
5
6
7
Pleopods
ABDOMEN
PLEOSOME
UROSOME
tooth
Telson
Uropods
Epimeron
peraeon plate 2
copulatory notch
coxa
♀
basis
carpus
ischium
merus
Gnathopod 1
Brood setae
(tip, enlarged)
Brood plate
coxal gill
propod
dactyl
carpal lobe
Gnathopod 2
sternal gill
merus
carpus
propod
Peraeopod 7
peduncle
ramus
Uropod 3
Telson
coupling spines
inner ramus
outer ramus
Pleopod 1
incisor
lacinia
right M.
Gnathopod 1
epistome
labrum
Upper lip
Lower lip
left M.
molar
Mandible
palp
outer plate
inner plate
Maxilla 1
Maxilla 2
Maxilliped

Figure F.1 **Outline of Body, Appendages, and Mouthparts of *Hyalella azteca* (updated from Bousfield, 1973)**

Hyalella azteca (Saussure, 1858)

Figure F.1: Diagnosis (modified from Bousfield, 1973)

Body Length: Male, to 8 mm; female, to 6 mm. Body small, dorsally mucronate on pleon segments 1 and 2, occasionally on 3, or smooth (form *inermis*). Coxal plates very deep, 4th largest; lower margins lightly and evenly spinose. Head, eye subovate, black, slightly larger in male.

Antenna 1, peduncular segments 1 and 2 subequal, flagellum 8-10 segmented. Antenna 2, peduncle slender, segment 5 longer than 4; flagellum 9-10 segmented. Maxilliped, palp segment 2 wider than long, exceeding outer plate.

Gnathopod 1 (male), propod shorter and less deep than carpus, expanding distally; palm oblique, convex. In female, propod narrow, short; palm vertical, convex. Gnathopod 2 (male), propod very large, distally broadest; posterior margin slightly concave; palm convex, with large low tooth near hinge; carpal lobe deep. In female, propod slender, elongate, expanding distally; palm short, convex, vertical.

Peraeopods 3 and 4, posterior margins of segments 5 and 6 with 3-5 short, stout spines. Peraeopods 5-7, basis broadly expanded, posterior margin with 4-10 weak serrations; segments 5 and 6 lacking posterior marginal spines or setae. Abdominal side plates (epimera) 2 and 3, hind corners sharply subquadrate, not produced.

Uropods 1 and 2, both rami with two slender marginal spines. Uropod 3, ramus and peduncle subequal in length, apex with long spine(s). Telson, apex rounded, with two slender wide-set spines.

Coxal gills on peraeopods 2-6 normal, sac-like, smallest on 6. Paired sternal gills at bases of peraeopods 3-5 and 7 are regular in form, not elongate or strongly curved.

With respect to other known North American species of the genus *Hyalella*, *H. azteca* (Saussure) differs in usually possessing a single postero-dorsal tooth or mucronation on each of pleon segments 1 and 2 (occasionally also on 3), and in the relatively elongate, narrow form of the propod and carpus of gnathopod 2 in the female, among other items.

(4) uropod 3 that has only a single short ramus;

(5) telson lobes short or fused (plate-like);

(6) coxal gills located on peraeopods 2-6 only (lacking on peraeopod 7); and

(7) brood plates (female) that are broad, and marginally fringed with short, curl-tipped setae.

H. azteca is a typical member of family Hyalellidae in having the following character states, in combination:

(1) antenna 1 longer than the peduncle of antenna 2;

(2) maxilliped palp with strong dactyl;

(3) gnathopod 2 (female) regular (not mitten-shaped);

(4) lower margin of peraeon 2 (female) with incised "copulatory notch";

(5) peraeopod distal segments not broadened or otherwise modified for burrowing;

(6) pleopods normal (not reduced, modified, or vestigial);

(7) telson entire, plate-like;

(8) coxal gills regular, unmodified; and

(9) sternal gills present variously on inner coxal margin of peraeopods 3-7.

Behaviourally, hyalellids appear to be incapable of jumping in air, as are all members of family Talitridae and many members of family Hyalidae.

With respect to other genera within family Hyalellidae, the genus *Hyalella* differs from *Parhyalella* in possessing sternal gills and a palp on maxilla 1. *Hyalella* differs plesiomorphically from the three austral genera (of chiltoniins, above) in having (in males) sexually mature unmodified pleopods, and a distinct posterior carpal lobe on gnathopod 2.

Distribution and Ecology

According to Bousfield (1958, 1973) and de March (1978), *H. azteca* has been recorded in North America from central Mexico north to about the tree line in Canada and Alaska, and, continent-wide, in virtually all permanent fresh waters that attain a regular summer surface temperature of 10°C or higher. Ecologically, the species prefers fresh waters that are somewhat hard or alkaline, with a normal pH range of 6.0 to 8.0. However, the species has also been found regularly in the upper (tidal) portions of coastal marine estuaries where salinities might reach 2 to 3 ‰ or higher, and in some alkaline lakes where total hardness might exceed 200 mg/L and brine shrimps co-exist (e.g., in some Quill Lakes of Saskatchewan).

With respect to water flow and substrate preferences, the species usually occurs abundantly in lentic waters or ponds where vegetative (especially algal) growth provides food and cover. It is found less frequently in streams and other lotic environments, especially where the bottom consists of uniformly-fine sediments that do not provide protective cover and/or organic food supply.

With respect to respiration, gaseous exchange takes place mainly through the paired coxal gills of peraeopods 2 to 6. Tolerance of low levels of dissolved oxygen and of high levels of carbon dioxide and decomposition gases is apparently higher in this species than in most other North American freshwater amphipods. The paired sternal gills (at the bases of peraeopods 3,4, 5, and 7) are believed to be mainly osmoregulatory in function, and might facilitate tolerance of a wide range and rapid fluctuation of ionic content within the aquatic medium. Sternal gills might also be partly respiratory in function.

Life Cycle and Reproductive Behaviour

The life cycle of *H. azteca* is essentially annual (Cooper, 1965; Strong, 1972; Conlan and Hendrycks, pers. commun.[45]). In spring, when water temperatures have continuously exceeded 10°C, the overwintering female produces a large clutch of up to 30 eggs. Following hatching and release of juveniles from the brood pouch or marsupium, the female continues to mate and produce further broods. Because of higher ambient summer water temperatures, these later broods occur at more frequent intervals, but clutch sizes tend to be smaller. The newly-hatched juveniles pass through 5 to 6 further instars, or growth stages, before reaching maturity. The spring-spawning females die before onset of the second winter, but late-hatching summer broods comprise the succeeding overwintering population.

With respect to reproductive behaviour, in primitive ("natant" or free-swimming) amphipod superfamilies, pelagic males search out and mate with females freely in the water column, often with a cyclic periodicity. As a member of superfamily Talitroidea, however, *H. azteca* is classified with the reproductively-advanced "reptant" or "bottom-crawling" clade of gammaridean amphipods (Bousfield, 1992; Bousfield and Staude, 1994). In these groups, using a process known as pre-amplexing, precopulation, or mate-guarding (Borowsky, 1984; Conlan, 1990, 1991), males attach themselves dorsally to females, usually by means of their tactile and prehensile first gnathopods. In *Hyalella*, the dactyl of the first gnathopod of the male fits into a special "copulatory notch" (on the lower margin of peraeonal plate 2; see Figure F.1) on both sides of the female's body, leaving the large second gnathopods free to rotate the female into a suitable carrying position and/or to fend off other males. In this "riding" position the male and female remain together, for several hours or days, until the female's next moult. Mating (i.e., amplexus, transfer of sperm) takes place immediately thereafter, on or in bottom substrata, in a short period of time, often within a few seconds, after which the pair separates permanently. During the brief post-exuvial period, the female is especially vulnerable to predation, occasionally by males of competing (or even the same) species, as has been shown in the case of some gammaroideans.

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[45] Canadian Museum of Nature (Ottawa, Ontario).

Appendix G

Procedure for Preparing YCT[46] Food for *Hyalella azteca*

Preparing Yeast

1. Add 5.0 g of dry yeast, such as Fleischmann's™, to 1 L of deionized water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow and Cerophyll preparations presented subsequently. Discard excess material.

Preparing Cerophyll™ (Dried, Powdered Cereal Leaves)

1. Place 5.0 g of dried, powdered Cerophyll™, cereal leaves, alfalfa leaves, or rabbit pellets[47] in a blender.
2. Add 1 L of deionized water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

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[46] Mixed ration consisting of yeast, Cerophyll™ (or acceptable substitute), and trout chow (or acceptable substitute). Taken from USEPA (1989) and USEPA (1994a).

[47] Cerophyll™ can be purchased from Ward's Natural Science Establishment Inc., P.O. Box 92912, Rochester, NY 14692-9012 (716-359-2502). Suitable substitutes for Cerophyll™ include dried, powdered cereal leaves, alfalfa leaves, or rabbit pellets (USEPA, 1994a). Cereal leaves are available from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 (800-325-3010). Dried, powdered alfalfa leaves can be obtained from health food stores, and rabbit pellets are available at pet shops.

Preparing Digested Trout Chow[48]

1. Preparation of trout chow requires one week. Use starter or No. 1 pellets[49].

2. Add 5.0 g of trout chow pellets to 1 L of deionized (Milli-Q™ or equivalent) water. Mix well in a blender and pour into a 2-L separatory funnel. Digest before use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation should be replaced during digestion. Because of the offensive odour usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

3. At the end of the digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine-mesh screen (e.g. Nitex™, 110 mesh). Combine with equal volumes of supernatant from Cerophyll™ and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the remaining particulate material.

Preparing Combined yeast-Cerophyll™-trout chow (YCT) Food

1. Thoroughly mix equal (e.g., 300 mL) volumes of the three foods as previously described.

2. Place aliquots of the mixture in small (50 to 100 mL) screw-cap plastic bottles.

3. Ideally, food should be stored at 4°C and used within two weeks of preparation. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is to be used for a maximum of one week. Do not store YCT frozen for more than three months.

4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7 to 1.9 g solids/L.

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[48] USEPA (1994a) indicates that a commercial flaked fish food such as Tetra-min™ may be substituted for trout chow.

[49] Suppliers of trout chow include Zeigler Bros. Inc., P.O. Box 95, Gardners, PA 17324 (phone 717/780-9009); Glencoe Mills, 1011 Elliott, Glencoe, MN 55336 (612/864-3181); and Murray Elevators, 118 West 4800 South, Murray, UT 84107 (800/521-9092).

Appendix H

Logarithmic Series of Concentrations Suitable for Toxicity Tests[50]

Column (Number of concentrations between 10.0 and 1.00, or between 1.00 and 0.10)[51]

1	2	3	4	5	6	7
10.0	10.0	10.0	10.0	10.0	10.0	10.0
3.2	4.6	5.6	6.3	6.8	7.2	7.5
1.00	2.2	3.2	4.0	4.6	5.2	5.6
0.32	1.00	1.8	2.5	3.2	3.7	4.2
0.10	0.46	1.00	1.6	2.2	2.7	3.2
	0.22	0.56	1.00	1.5	1.9	2.4
	0.10	0.32	0.63	1.00	1.4	1.8
		0.18	0.40	0.68	1.00	1.3
		0.10	0.25	0.46	0.72	1.00
			0.16	0.32	0.52	0.75
			0.10	0.22	0.37	0.56
				0.15	0.27	0.42
				0.10	0.19	0.32
					0.14	0.24
					0.10	0.18
						0.13
						0.10

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[50] Modified from Rochinni *et al.* (1982).

[51] A series of five (or more) successive concentrations should be chosen from a column. Midpoints between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by weight (e.g., mg/kg) or weight-to-volume (e.g., mg/L). As necessary, values can be multiplied or divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations should not be used, since such will provide poor resolution regarding the confidence limits surrounding any threshold-effect value calculated.